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Hexachlorophene Effects on the Rat Brain

Study of High Doses by Light and Electron Microscopy

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Hexachlorophene Effects on the Rat Brain

Study of High Doses by Light and Electron Microscopy

Renate D. Kimbrough, MD, and Thomas B. Gaines, Chamblee, Ga

Hexachlorophene was fed to female adult Sherman strain rats at 500 ppm (25 mg/kg/day) for 14 and 10 weeks in two consecutive studies. After two weeks on the diet containing hexachlorophene, the rats developed leg weakness which progressed to paralysis in three to five weeks. After ten weeks on these diets in the second study, hexachlorophene was discontinued; the rats regained almost complete recovery of leg function within six weeks. The brains of the rats that had ingested hexachlorophene weighed significantly more than those of controls and returned to normal in six weeks. The lesions produced by hexachlorophene in the rat brains represented cerebral edema limited to the white matter, which resembles diffuse spongy degeneration of the white matter of the brain described in infants.

ALTHOUGH hexachlorophene is used extensively as a germicidal agent[1] and is now recommended for use in agriculture as a broad spectrum fungicide and bactericide,[2] little is known about its oral toxicity. In the course of toxicity studies conducted in our laboratory, rats on a diet containing high concentrations of hexachlorophene developed flaccid paralysis of their hindquarters as a result of the effect of the technical hexachlorophene compound on the brain and spinal cord. We will describe the brain lesions produced by the hexachlorophene and the recovery of the rats after exposure to hexachlorophene had been discontinued. The results of further studies of the toxicity of hexachlorophene in rats will be published later.

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Procedure

Sherman strain adult female rats were used. They were caged individually and weighed weekly. Hexachlorophene, US Pharmacopoeia, was fed to rats at 500 ppm in ground laboratory chow in two different studies (10 rats in the first study and 12 rats in the second). An equal number of rats of similar age and body weight were kept as controls in each study and fed the same diet without hexachlorophene. Water, as well as the diets, was given ad libitum to all rats. Based on measurements of food consumption during weeks 1, 2, 7, and 12 in the first study and weeks 1, 2, and 7 in the second study, the rats who were fed hexachlorophene received a mean dose of 25 mg/kg/day of the compound. The rats in the first study were fed the hexachlorophene-treated diet for 14 weeks and then were killed. The rats in the second study were fed the treated diet for ten weeks and then the hexachorophene was discontinued. In the latter study, three of the control rats and three of the rats fed hexachlorophene were killed at the time that the hexachlorophene was discontinued, and three more of each group were killed at 2, 6, and 12 weeks thereafter, respectively.

Complete autopsies were performed on all rats of the first study. Only the brains were

examined in the second study. The brains to be examined by light microscopy were fixed in Bouin's fluid; all other organs were fixed in 3.7% to 4% buffered formaldehyde solution (10% buffered formalin). These tissues were stained with hematoxyin-eosin, and selected brain sections were also treated with Weil's stain for demonstration of myelin. Brain tissue to be used for study with the electron microscope was fixed in phosphate-buffered 5% glutaraldehyde for two hours and was postfixed in 1% osmium tetroxide, dehydrated, and embedded in resin (Maraglas). For various clinical reasons, fixation by perfusion was not used. Sections were cut with a glass knife and examined under an electron microscope. An area of the white matter of the cerebral hemispheres was selected for this purpose.

Results

In both studies, after the rats were exposed to the hexachlorophene-treated diets for about two weeks, they showed signs of nervousness and weakness in the hindquarters. This weakness progressed to paralysis within three to five weeks of hexachlorophene exposure in eight of the rats in the first study and in ten of the rats in the second study. One rat with paralysis died in the first study on the 58th day of exposure. Two weeks after the hexachlorophene was discontinued in the second study, function began to return to the hindquarters, and within six weeks the rats that had not been killed had recovered almost completely. At autopsy it was found that the weights of the brains of the rats receiving hexachlorophene in the first study ranged from 2.33 to 2.75 gm (average, 2.60 gm; SE ± 0.04). The weights of the brains of the controls ranged from 1.86 to 2.10 gm (average, 1.99 gm; SE ± 0.02). This increase in brain weight in the exposed group was statistically significant ($P < 0.001$).

In the second study, the increase in brain weight was again noticed. After the animals had been off the diet for six weeks, the brain weights had returned to normal. At autopsy it was found that the brains of the poisoned rats were very soft, although their surfaces were normal. On cross section after fixation, the brains showed occasional small holes up to 1 mm in diameter; the white matter of the cerebrum and cerebellum was widened, generally depressed, and finely porous in some areas. All other organs except the central nervous system were both grossly and microscopically normal in the first study and, therefore, were not examined in the second study. The brains of the control rats were also both grossly and microscopically normal. The gray matter of the brains of the rats fed hexachlorophene was normal microscopically. Examination of the white matter of the brain with the light microscope revealed a meshwork of cystic spaces of different sizes surrounded by thin strands of fibers and normal-appearing glia cells. These areas were much wider than normal, which was particularly evident in the cerebellum (Fig 1, control, and Fig 2, treated). Using Weil's stain, a few dark fibers were observed in the trabeculae surrounding the cystic spaces, although the cystic spaces themselves were empty. The same meshwork of cystic spaces was also observed in the white matter of the spinal cord of these rats.

Examination of the areas of the white matter under the electron microscope showed large empty cystic spaces lined by fragments of myelin and surrounded by some nomal-appearing brain tissue. These spaces occasionally contained a slight amount of cellular debris (Figs 3 and 4, treated). The myelin surrounding a number of axons showed signs of degeneration; portions of it had separated and formed small globules or circles. The brains of the animals that had been killed two weeks after their exposure to hexachlorophene had been discontinued showed the same vacuolated structure of the white matter as the brains of the rats that had not been allowed to recover at all.

The brains of rats which were killed six weeks after exposure to hexachlorophene had been discontinued were grossly normal. When they were examined under the light microscope and the electron microscope, a few cystic spaces were found to be present in the white matter of the brain.

The brain of three rats killed 12 weeks after exposure to hexachlorophene had been discontinued still showed a few cystic spaces in the white matter of the cerebrum and cerebellum by light microscopy. The brains of three control rats killed simultaneously were normal.

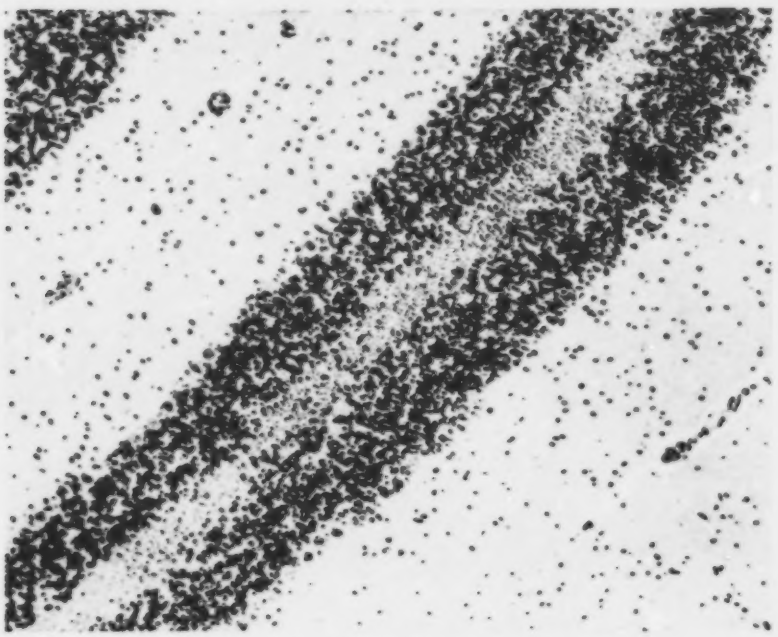
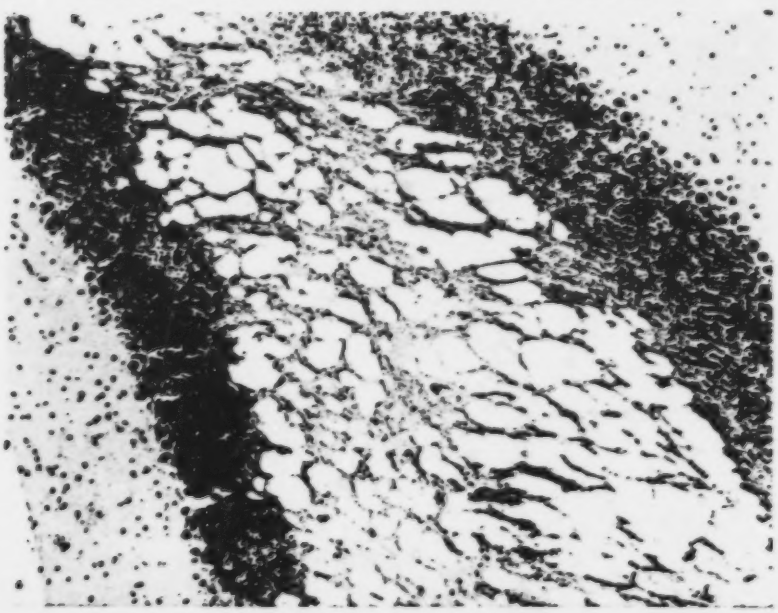


Fig 1.—Control rat cerebellum (hematoxylin-eosin, ×100).

Fig 2.—Section of cerebellum from rat fed 500 ppm hexachlorophene for 14 weeks. Note cystic spaces in white matter of the cerebellum and also widening of area of white matter (hematoxylin-eosin, ×100).



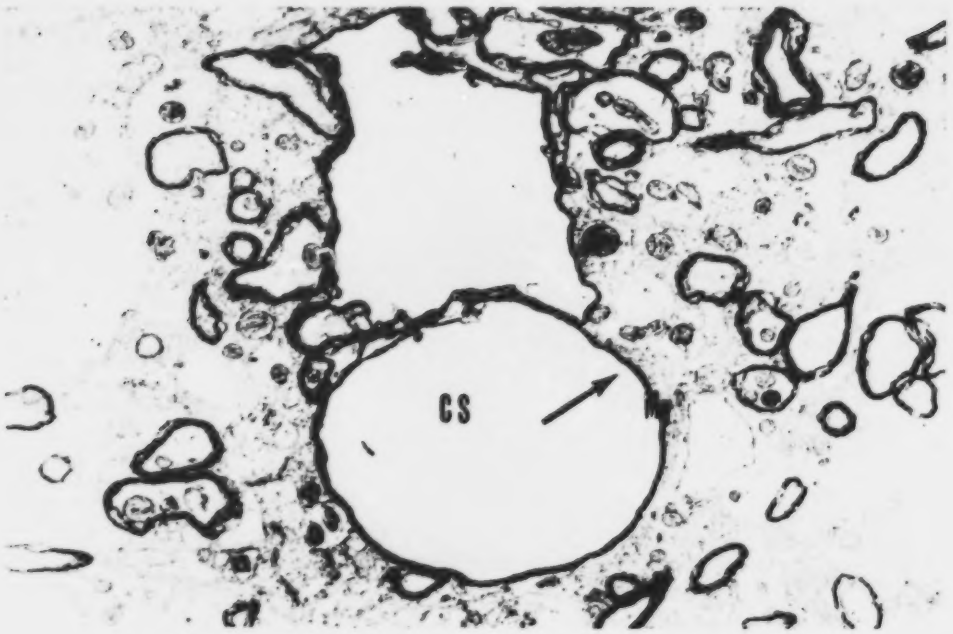


Fig 3.—Section through area of white matter of cerebrum of rat fed 500 ppm hexachlorophene for ten weeks. My, myelin; CS, large cystic spaces illustrated in Fig 2 and lined by a thin layer of myelin (arrow) (lead citrate, ×9,000).

Fig 4.—Higher magnification of Fig 3 illustrating the myelin lining the cystic spaces (arrow) (lead citrate, ×37,000).

Comment

The brain and spinal lesions produced by hexachlorophene were limited to the white matter of the central nervous system and consisted of cystic spaces that appeared empty. Water content of the rat brains was not determined in this study, but the cystic spaces of the brain were most likely produced by fluid accumulation. Strangely, it occurred only in the white matter and did not affect the gray matter at all. The ultrastructural findings suggest that the axons of myelinated nerves are either compressed or destroyed by fluid accumulation in the myelin sheaths. The myelin sheaths are subsequently dilated and secondarily affected. Similar conditions have been described in infants and have been referred to as spongy degeneration of the white matter.[3] Magee et al[4] described the experimental production of edema in the central nervous system of the rat by triethyl tin compounds. The description of the brain lesions in the rats they studied is very similar to the lesions in the rats of our study. These authors found an increased water content in the affected brains, and the formation of cystic spaces in their animals was limited to the white matter of the central nervous system.

Gump[1] recently reviewed the toxicological properties of hexachlorophene. In his report, he makes reference to several cases of human poisoning in which a commercial preparation of hexachlorophene had been mistaken for milk of magnesia and ingested. A case of hexachlorophene poisoning after absorption of the material through the skin of an infant was reported with clinical signs of neurological involvement. The mother applied hexachlorophene as a lotion to the infant after bathing him and left it on his skin. After four days of this treatment, excoriations appeared on the buttocks and face and there were slight twitchings of arms, legs, and face, which progressed to convulsions.[5]

It must be remembered that the amount of hexachlorophene ingested by the rats in our study was large (25 mg/kg/day for 14 weeks). In the toxicity studies presently in progress in our laboratory, 100 ppm (5.0 mg/kg/day) of hexachlorophene fed in the diet for 14 weeks did not cause symptoms of paralysis. Microscopic examination of the brains of these animals revealed a few cystic spaces in the white matter. This may be considered a borderline lesion, and we can speculate that hexachlorophene fed at concentrations of less than 100 ppm for 14 weeks will not affect the brain of adult female Sherman strain rats. Rats fed 500 ppm hexachlorophene for ten weeks recover from paralysis if the exposure to hexachlorophene is discontinued. Even after 12 weeks of recovery, some evidence of the effect of hexachlorophene can still be observed microscopically in the white matter of the brain. Thus, indiscriminate and unnecessary use of hexachlorophene that results in significant internal exposure should be discouraged. Contamination of food with hexachlorophene should be avoided until the toxicity of hexachlorophene has been further evaluated.

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DDT and DDE Residues in Blood From Children, South Carolina—1970

Julian E. Keil,[1] William Weston, III,[2] C. Boyd Loadholt,[2]
Samuel H. Sandifer,[1] and James J. Colcolough [4]

ABSTRACT

DDT and DDE residue levels in blood plasma from 192 children in South Carolina, ages 6-9 years, indicated that Negro children had levels two to three times higher than white children. DDT residues averaged 18.4 ppb in Negroes and 6.7 ppb in whites; DDE values for these two races were 55.6 ppb and 24.8 ppb, respectively. White males in this group also had significantly higher levels of both compounds than white females. From the data in this study, baseline levels for a high-risk pediatric group, usually prone to pesticide poisoning, were established.

Introduction

In 1969, the S. C. Community Pesticide Study measured pesticide residues in plasma from 800 persons in Charleston County; the subjects had been selected by sex, race, residence, and 10-year age groupings (6). The outstanding findings of this study were: (1) Negroes had much higher levels of DDT and its metabolites than whites, and (2) children between the ages of 5 and 10 years had significantly higher DDT DDE levels than the rest of the population studied and, in some instances, as high as DDT formulators.

The present study, partially reported here, was begun with several purposes in mind: (1) to confirm earlier findings of the South Carolina investigators of higher DDT and DDE levels among children and Negroes; (2) to establish new baselines for young children who are prone to poisoning, because analytical methods have changed since the earlier work; and (3) to explore reasons for the age and racial differences previously reported. This last endeavor is still under study.

Sampling Methods

A total of 192 apparently healthy children, ages 6-9 years from public and private schools in Charleston, S. C., were stratified by race (nonwhites were almost exclusively Negro), sex, residence, and 1-year age spans. After approval by school authorities, written parental permission was obtained for blood sampling of the children. Venous blood samples were collected in heparinized vacutainers. Residue values for this group were compared with those of control subjects of the S. C. Community Pesticide Study.

Analytical Procedures

Plasma extraction was carried out by a modified method of Dale, Curley, and Cueto (*1*), as recommended by the Perrine Primate Research Branch Laboratory in Perrine, Fla. Two milliliters of plasma were placed in a 16 x 125 mm round-bottom culture tube fitted with screw caps, size 15-415 with Teflon-faced liners; 6 ml of nanograde hexane were added; and the mixture was rotated on a slow speed rotating mixer at 50 rpm for 2 hours. The formation of emulsions was infrequent; when they did occur, centrifugation was used to separate the layers. Following the 2-hour rotation period, a 5-ml aliquot of the hexane layer was quantitatively transferred to a 10-ml graduated concentrator tube. The degree of concentration or dilution that was necessary was determined by a preliminary analysis of the 5-ml aliquot. If concentration was necessary, one 3-mm glass bead was added, and a modified micro-Snyder column was attached. The extract was evaporated on a steam bath to a volume of about 200 μl. The tubes

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were allowed to cool for about 5 minutes, the micro-Snyder column was removed, and the sides of the tube and column joint were rinsed down with hexane. The final volume was adjusted to 500 μl for subsequent analysis.

No cleanup procedure was required or utilized. A 5 μl portion was then injected into a MicroTek 220 gas chromatograph equipped with a tritium foil electron capture detector. All injections were off-column injections. All samples were analyzed on the OV-17/QF-1 column, and the residues were confirmed on the SE-30/QF-1 column. It was necessary in almost all cases to run several sample extract chromatograms of various concentrations to achieve reasonable approximation of peak sizes with those of the standard mixture. The samples were stoppered and mixed on a Vortex mixer for about 30 seconds after each dilution before injection.

GAS-LIQUID CHROMATOGRAPHY

The operating parameters for gas chromatographic analysis were as follows:

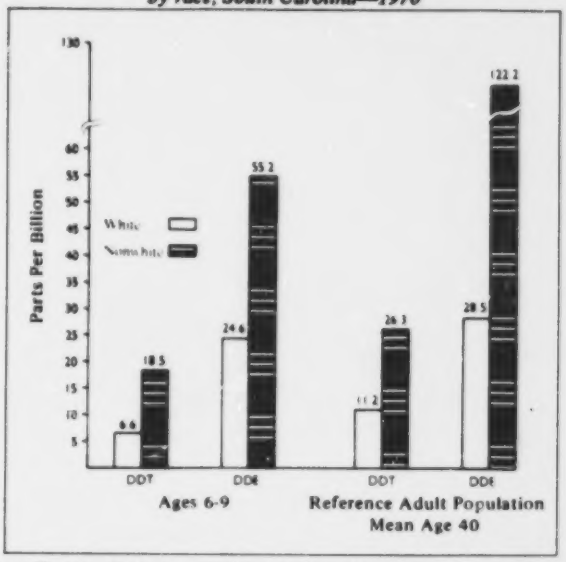
Columns:	1.5% OV-17 and 1.95% QF-1 on Chromosorb W, DMCS, HP, 100/120 mesh; 4% SE-30 and 6% QF-1 on Chromosorb W, DMCS, HP, 100/120 mesh	
Temperatures:	Injection chamber	225° C
	Column	200° C
	Transfer block	235° C
	Detector	205° C
Nitrogen flow:	OV-17/QF-1	60 ml/min
	SE-30/QF-1	80 ml/min

All qualitative retention times were based on the retention time of aldrin. Quantitation of pesticide residues was based on relative peak areas which were supplied by an Infotronics Chromatograph Integrator Model CRS-101. Recovery, based on the addition of a known quantity of *p,p'*-DDE and *p,p'*-DDT, ranged from 95.0 to 98.6%. Residue results were not corrected for recovery. The minimum reporting limit for *p,p'*-DDE was 1.0 ppb and for *p,p'*-DDT, 2.0 ppb. No polychlorinated biphenyls were detected in any of the samples analyzed. A least squares analysis was used to estimate and test differences between strata means.

Results and Discussion

Results of previous studies indicating unusually high DDT/DDE levels in preadolescents were not confirmed. As shown in Fig. 1, children in the 6-9 age group had lower levels than did their adult counterparts (control subjects of the S. C. Community Pesticide Study). This is at variance with earlier work by Finklea *et al.* (*6*), but consistent with results reported by Watson *et al.* (*7*). Improvements in analytical techniques since the earlier study (*6*) may have reduced variances in results and accounted for the lower levels.

FIGURE 1.—*Mean plasma levels of p,p'-DDT and p,p'-DDE in a pediatric group and adult reference group by race, South Carolina—1970*



DDT and DDE were two to three times higher in the Negro than in the white group. This difference was evident in the juvenile cohorts as well as in the adult reference group (Fig. 1). This study confirms racial differences in DDT/DDE residue levels between whites and nonwhites reported by Davis (*2,3*), Edmundson (*4,5*), and Finklea (*6*).

White males had significantly higher levels of both compounds than white females (Table 1). No significant differences in levels of DDT or DDE were apparent when sex and race were evaluated according to the place of residence of subjects (Table 2); thus, values in Table 1 may be used to estimate "normal" ranges for this high-risk pediatric age group, unusually prone to pesticide poisoning.

Table 3 lists DDT and DDE levels by 1-year age groups and indicates no significant increase or decrease in levels with age.

TABLE 1.—*DDT and DDE residue levels in plasma from 192 children, ages 6-9 years, by race and sex, South Carolina—1970*

Compound	White*			Nonwhite		
	Male (N=50)	Female (N=46)	SD[1]	Male (N=45)	Female (N=51)	SD[1]
DDT (ppb)	8.5	4.8	6.5	16.9	19.7	11.0
DDE (ppb)	29.9	19.2	14.2	55.2	55.9	29.5

[1] Pooled standard deviation for sex within races.
* Differences between levels in white males and females significant at $P < .01$.

TABLE 2.—*DDT and DDE residue levels in plasma from 192 children, ages 6-9 years, by residence, race, and sex, South Carolina—1970*

Race and Sex	Mean Residue Levels (ppb)			
	Urban		Rural	
	DDT	DDE	DDT	DDE
White Males	7.8 (N=27)	28.2	8.9 (N=23)	31.0
White Females	4.8 (N=24)	16.9	4.7 (N=22)	21.8
Nonwhite Males	16.5 (N=25)	54.5	17.7 (N=20)	54.5
Nonwhite Females	19.1 (N=27)	49.2	20.5 (N=24)	63.5

TABLE 3.—*DDT and DDE residue levels in plasma by age of 192 school children, Charleston County, S. C.—1970*

Age (Years)	Mean Residue Levels (ppb)	
	DDT	DDE
6 (N=35)	14.5	41.5
7 (N=35)	13.4	43.4
8 (N=42)	11.9	38.1
9 (N=70)	11.7	38.5

See Appendix for chemical names of compounds discussed in this paper.

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Induction of Adenofibrosis and Hepatomas of the Liver in BALB/cJ Mice by Polychlorinated Biphenyls (Aroclor 1254) [1]

Renate D. Kimbrough, *M.D.*,[2,3] *and* Ralph E. Linder [2,4,5]

SUMMARY—Two groups of 50 BALB/cJ inbred male mice were fed 300 ppm of a polychlorinated biphenyl, Aroclor 1254, in the diet for 11 and 6 months, respectively. The 6 months' feeding was followed by 5 months' recovery. Two additional groups of 50 mice each were fed plain chow. All 22 surviving mice fed Aroclor 1254 for 11 months had greatly enlarged livers representing 25% of their body weight, whereas those fed the experimental diet for 6 months only had slightly, but significantly, enlarged livers. Adenofibrosis was observed in all 22 livers of mice fed Aroclor 1254 for 11 months but not in the other groups. Of the 22 mice fed 300 ppm Aroclor 1254 for 11 months, 9 had 10 hepatomas measuring 0.1–1.5 cm in diameter. One of 24 surviving mice fed Aroclor 1254 for only 6 months, followed by a control diet for 5 months, had a hepatoma 0.3 cm in diameter. No controls had hepatomas.—J Natl Cancer Inst 53: 547–552, 1974.

IN PREVIOUS STUDIES with Sherman rats, prolonged feeding of polychlorinated biphenyls (PCB's) Aroclor 1254 particularly, but also Aroclor 1260, induced a lesion, classified as adenofibrosis, in the liver (*1*, *2*). Edwards and White (*3*) and Stewart and Snell (*4*) described this lesion in detail.

On gross inspection adenofibrosis, also called cholangiofibrosis, is firm and grayish white and has a pitted or granular surface. Microscopically, it consists of fibrosis and proliferated, atypical-appearing glandular epithelium that forms cysts and ducts. To ascertain whether this lesion could also be produced in mice, we conducted the present study.

MATERIALS AND METHODS

Two hundred 5- to 6-week-old BALB/cJ inbred male mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. According to information from the Jackson Laboratory (*5–7*), this strain has a very low incidence of spontaneous tumors, particularly hepatomas.

The mice were distributed randomly into 4 groups of 50. They were tagged individually and caged in groups of 5. The mice were weighed every 2 weeks for the first 6 weeks and thereafter every 4 weeks. Two groups of 50 mice each were fed 300 ppm Aroclor 1254 in ground Purina Laboratory Chow. After 6 months of exposure, 1 group was given plain ground chow, while the other group consumed the experimental diet for 11 months. The 2 additional groups were controls and were fed plain ground laboratory chow throughout the 11-month study. Food consumption was measured during the 2d and 6th week of exposure and thereafter every 8 weeks in the group exposed to the experimental diet for 11 months and in 1 control group.

The Aroclor 1254 (Lot AK-38, from Monsanto Chemical Co., St. Louis, Mo.) was first dissolved in ether and then mixed with cornstarch. The ether was allowed to evaporate, and the Aroclor 1254–cornstarch mixture was added to increasing amounts of ground chow.

Early in the experiment, some mice were lost because of fighting. All mice, including controls, were caged in pairs 2 months after onset of the experiment. Since most mice that died did so early in the study and often showed a great deal of autolysis, they were not included in the final evaluation of the liver pathology. Organs of all mice that were killed were inspected grossly for lesions; the livers were removed, weighed, fixed, and then carefully inspected grossly for hepatomas. Any abnormal-appearing tissue was also examined microscopically. The tissues were fixed in 4% buffered formaldehyde, stained with hematoxylin and eosin, and examined with the light microscope.

RESULTS

In the control group, the food consumption ranged from 167 to 198 g/kg body weight/day. In the group fed Aroclor 1254 for 11 months, it ranged from 159 to 171 g/kg body weight/day, which resulted in a mean dosage level of Aroclor 1254 of 49.8 mg/kg/day. During the first 4 months, 23 mice died in the 2 control groups and 20 in the 2 experimental groups. Table 1 gives the total survivors in the different groups, body and liver weights, and incidence of hepatomas. A slight, but statistically significant, increase in body weight was observed in mice receiving the experimental diet for 11 months. Compared to controls, these mice had large livers, representing 25% of their body weight. The surface of the liver of 9 of these mice contained nodules of varying sizes up to 1.5 cm in diameter. All livers of the mice that were killed in this group showed orange fluorescence under ultraviolet light, indicative of elevated porphyrin levels. The body weights of the mice fed Aroclor 1254 for only 6 months were comparable to those of the control groups at the time they were killed; but, when the treated diet was discontinued, they weighed an average of 3 g more than the controls.

Upon microscopic examination of the livers of the 34 and 24 surviving controls in the 2 groups, 45 livers were normal. A few times a liver cell showed a large, hyperchromatic nucleus or binucleation. The re-

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[4] *Present address:* Environmental Protection Agency, Research Triangle Park, N.C. 27711.

[5] Dr. H. L. Stewart kindly reviewed the slides and Dr. M. Deringer supplied the information on the spontaneous incidence of hepatomas.

TABLE 1.—*Body weights, liver weights, and hepatoma incidence in surviving mice fed Aroclor 1254 in the diet and in controls fed plain chow*

Dietary level (ppm)	Dosage level (mg/kg/day)	Time exposed to experimental diet	Total mice	Total survivors	Final body weight of survivors: arithmetic mean (g)	Liver weight: arithmetic mean (g)	Liver weight expressed as percentage of body weight	Hepatoma incidence* in survivors
0	0	0	50	34	30.7	1.77	5.8	0
0	0	0	50	24	30.9	1.78	5.8	0
300	49.8	11 months	50	22	33.8†	8.62†	25.5†	3(0.1–0.4 cm ø) 3(0.5 cm ø) 4(1–1.5 cm ø)
300	Not checked	6 months and 5 months' recovery.	50	24	31.5	2.37†	7.5†	1(0.3 cm ø)

*Determined when mice were 12 months old. ø=diameter.
†$P<0.001$. A *t*-test was used for the statistical evaluation.

maining 13 livers had focal, round-cell infiltrates and occasionally small areas of necrosis and fibrosis. All mice with round-cell infiltrates in the liver had small skin abscesses, usually near the groin, which appeared to be abscesses of the preputial gland.

Microscopic examination of the livers of the 22 survivors in the group receiving the experimental diet for 11 months (fig. 1) showed markedly enlarged liver cells. The nuclei of the hepatocytes were enlarged, hyperchromatic, and atypical. The cytoplasm was either smooth or vacuolated. Macrophages and Kupffer's cells contained a brown pigment. Slight, diffuse interstitial fibrosis and numerous, large extracellular hyaline bodies were also seen. Some livers had extensive areas of coagulation necrosis and fibrosis (fig. 2). A general pleomorphism was noted in a number of livers. Degeneration, necrosis, and atrophy of individual liver cells were also observed.

A few nuclei showed karyorrhexis. Some smaller cells and proliferation of the focal nodular type were also noted. The irregular outer surface of the liver with areas of retraction indicated that proliferation, atrophy, and regression had occurred. Occasional calcareous deposits ≈5–8μ in diameter were present. In each of the livers, several areas were seen where the parenchyma was replaced by glandular formations of proliferated epithelial cells which formed ducts and often produced mucus. These epithelial cells were surrounded by either sparse (fig. 3) or abundant connective tissue.

In addition to the above findings for all 22 livers of mice treated for 11 months (table 1), 10 hepatomas were found (fig. 4); of these, 3 measured 0.1–0.4 cm in diameter, 3 measured 0.5 cm, and 4 measured 1–1.5 cm. The tumors developed in a total of 9 mice. On gross inspection, they appeared as tan nodules. On microscopic examination, they consisted of well-differentiated hepatocytes that were relatively uniform and usually smaller than the surrounding liver cells. The tumors were well circumscribed and surrounded by compressed hepatic parenchyma or strands of fibrous tissue. The larger tumors contained areas where the sinusoids were dilated and filled with a pink-staining amorphous material. On gross inspection of the organs at autopsy, metastases were not seen. However, detailed screening for metastases including serial sectioning of lungs was not undertaken.

Most livers of the 24 mice surviving 6 months of dietary exposure to PCB's followed by a 5-month recovery had enlarged cells; often these liver cells had enlarged, atypical, hyperchromatic nuclei, although the livers at autopsy weighed only slightly more than those of the controls. Necrosis of individual liver cells was observed and some nuclei showed karyorrhexis.

The macrophages and Kupffer's cells usually contained a brown pigment. Many liver cells, particularly those in the periphery of the lobules, had vacuolated cytoplasm. The livers of about two-thirds of these mice showed slight-to-moderate diffuse, interstitial fibrosis. The outer surface of the livers still showed small indentations but not as pronounced as those in the group receiving PCB's for 11 months. Numerous extracellular hyaline bodies were observed in some

livers. In addition, many calcareous deposits, light in the center and darker in the periphery, were also seen (fig. 5). They were more frequent in this group than in the one fed PCB's for 11 months. A few livers showed bile duct proliferation. Only 1 liver contained a small hepatoma measuring 0.3 cm in diameter. The liver cells comprising the hepatoma were well-differentiated hepatocytes.

Except for the subcutaneous abscess formations in some mice and 1 sweat gland adenoma, no other findings of note were made in the mice of any groups.

DISCUSSION

Two significant findings were made in the present study: adenofibrosis of the liver and hepatomas. Thus adenofibrosis can also be induced in mice and does not occur solely in rats where it has been described previously (*1*, *3*). A similar lesion may occur in hamsters (*8*). In the mouse lesion, the epithelial component was much better differentiated and less pleomorphic than in the rat lesion (*1*). The pattern of the lesions and the interrelationship with fibrosis were the same. Ducts and cysts formed in areas of adenofibrosis in both the mouse and rat livers. Whether adenofibrosis is a precursor of a malignant lesion or occurs concomitantly with malignant lesions of the liver is still debated. In an excellent review, Stewart and Snell (*4*) concluded that the lesion showed no convincing evidence of a precancerous lesion. Reuber (*8*) postulated that cholangiocarcinomas would develop from adenofibrosis in rats as well as hamsters.

The mouse strain used in this study only rarely develops hepatomas spontaneously. Peters et al. (*5*) observed 6 hepatomas in a group of 2000 BALB/cCr mice, a subline from the Andervont subline at the National Cancer Institute; one would expect incidences similar to those observed in the BALB/cJ subline. Madison et al. (*6*) found 13 (0.62%) hepatomas in 2088 BALB/cCr mice 18 months of age. In the present study none of the controls, 1 of 24 mice fed PCB's for 6 months, and 9 of 22 mice fed PCB's for 11 months developed hepatomas.

Although the hepatomas were well differentiated, according to Andervont and Dunn (*9*), they represent potentially malignant lesions, a certain percentage of which can metastasize and be transplanted. From the appearance of the lesion, it is impossible to predict which tumor will be malignant. The size of the liver tumors cannot be used as a criterion. Quite possibly the tumors would have been larger and would have occurred more often had the mice been allowed to complete their lifespans.

A Japanese PCB, Kanechlor 400, was recently reported to have induced hepatomas in female Donryu rats (*10*), and Kanechlor 500, when fed at a dietary level of 500 ppm for 32 weeks, produced hepatomas in male dd mice (*11*). The dietary levels of PCB's in our study, as well as those of the Japanese studies, were high compared to levels that could be found in the normal human diet. Presently, temporary tolerance levels, ranging from 0.2 ppm in infant food to 5 ppm in fish and poultry, have been set by the U.S. Food and Drug Administration (FDA) under the authority of Section 406 of the Federal Food, Drug, and Cosmetic Act. The temporary tolerance level in poultry was set on a fat basis. The FDA concluded that the total daily food intake of PCB's should not exceed 1.4 mg/adult (*12*). The mice in the present study consumed daily ≈50 mg PCB's/kg body weight, which is 2500 times as much as the theoretical daily human consumption of 0.02 mg/kg body weight calculated by the FDA. The present study does not indicate whether lower levels would also induce hepatomas or whether a "no-effect level" for hepatomas could be established in rodents.

We have not previously observed calcareous deposits in livers of experimental rodents. The deposits we presently observed could represent calcification of small foci of tissue necrosis, particularly in areas where extracellular hyaline bodies were present.

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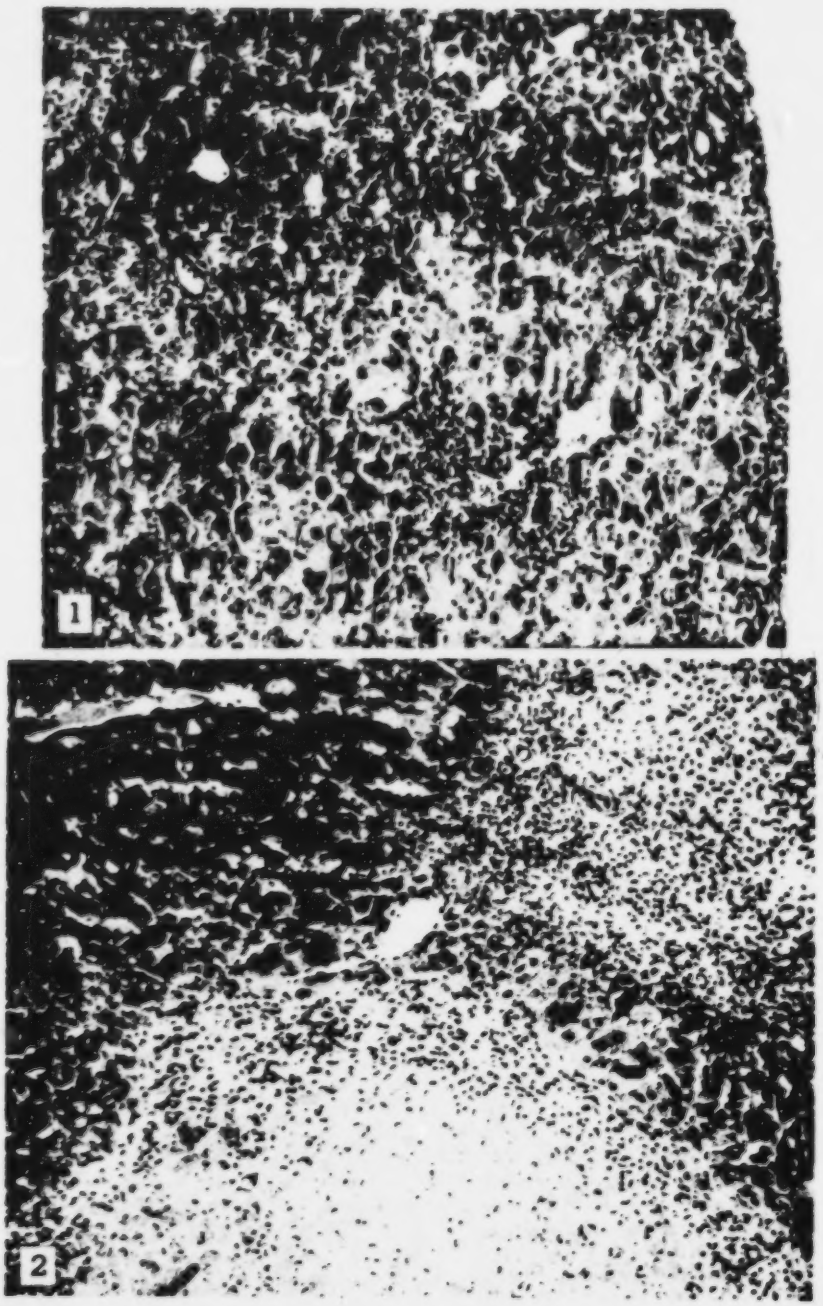


FIGURE 1.—Liver of mouse fed 300 ppm Aroclor 1254 for 11 months. *Note* **enlarged pleomorphic hepatocytes and interstitial fibrosis in some areas. Hematoxylin and eosin (H & E). × 100**

FIGURE 2.—Liver of mouse fed 300 ppm Aroclor 1254 for 11 months. *Note* **extensive coagulation necrosis and fibrosis. H & E. × 100**

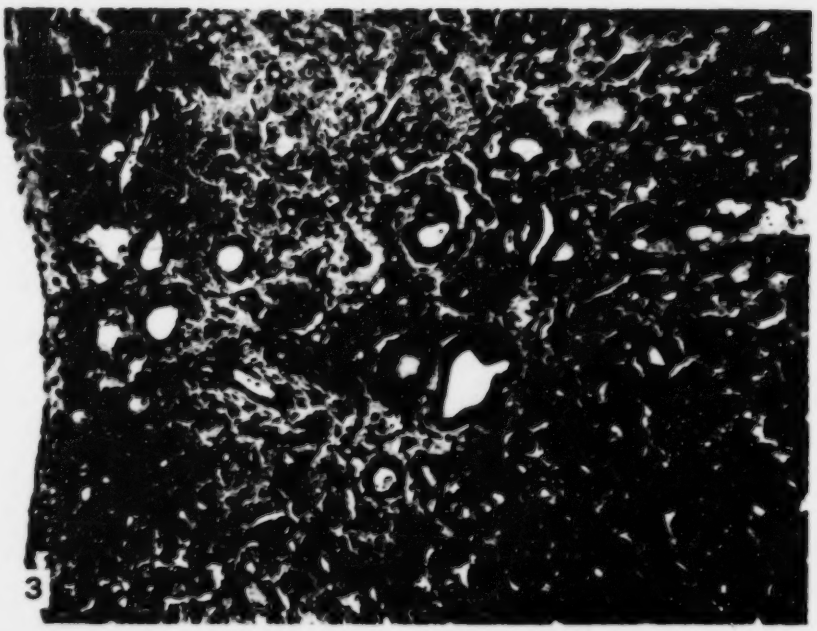
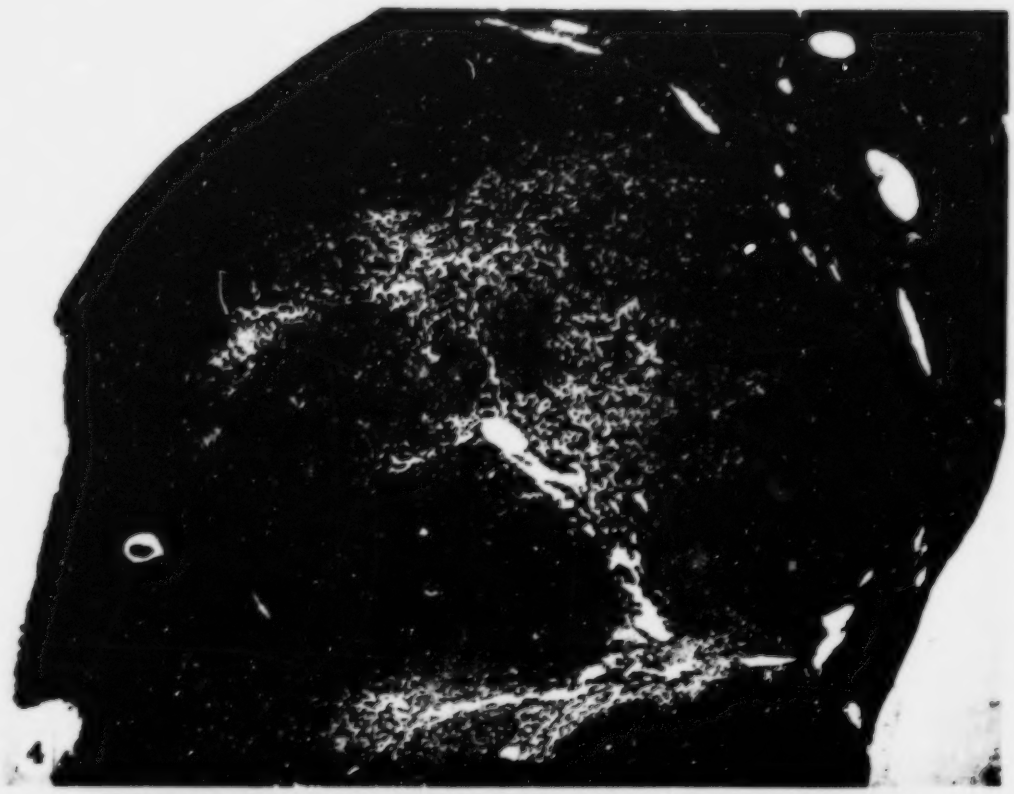


FIGURE 3.—Liver of mouse fed 300 ppm Aroclor 1254 for 11 months. *Note* epithelial cells forming ducts and surrounded by connective tissue. H & E. × 125



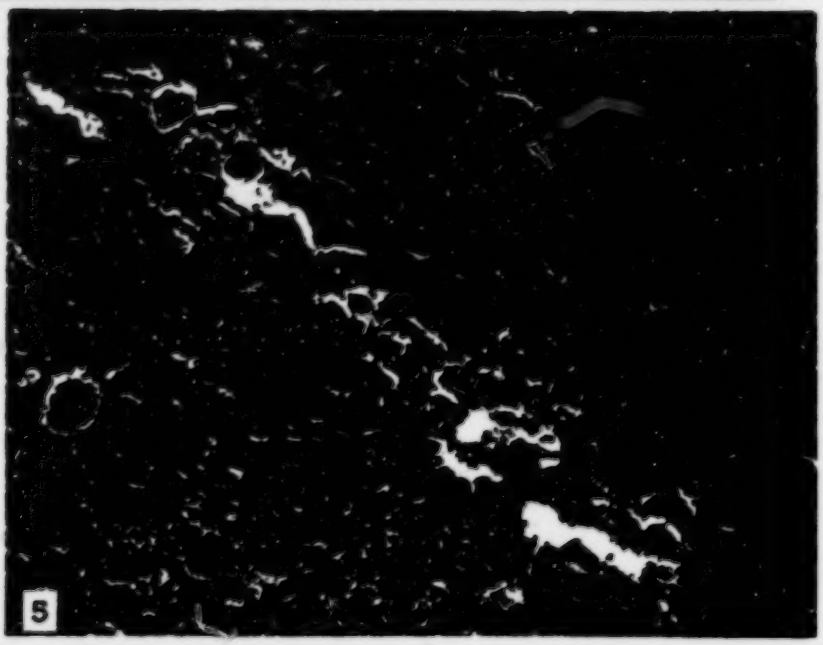


FIGURE 4.—Well-circumscribed hepatoma showing compressed hepatic parenchyma proper in the *upper right-hand corner*. H & E. × 20
FIGURE 5.—Liver of mouse fed Aroclor 1254 for 6 months, followed by recovery for 5 months. *Note* line of calcareous deposits traversing through *center* of section. H & E. × 125

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Brain-Acetylcholinesterase Inhibition in a Marine Teleost during Lethal and Sublethal Exposures to 1,2-Dibromo-2,2-dichloroethyl Dimethyl Phosphate (Naled) in Seawater[1]

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Brain-Acetylcholinesterase Inhibition in a Marine Teleost during Lethal and Sublethal Exposures to 1,2-Dibromo-2,2-dichloroethyl Dimethyl Phosphate (Naled) in Seawater. COPPAGE, D. L. AND MATTHEWS, E. (1975). *Toxicol. Appl. Pharmacol.* 31, 128–133. To determine if brain-acetylcholinesterase (AChE) inhibition in a marine teleost *Lagodon rhomboides* (pinfish) by an organophosphate pesticide (naled) is specific enough to diagnose anticholinesterase poisoning, brain-AChE inhibition by sublethal exposure in seawater was compared to brain-AChE inhibition caused by lethal exposure. A sublethal exposure did not inhibit brain-AChE as much as lethal exposure in periods of 24, 48, and 72 hr. Consistent levels of inhibition (84–89% inhibition) occurred when 40–60% of an exposed population of pinfish was killed. This correlation of brain-AChE inhibition with exposure and death in a fish population shows that brain-AChE measurements are of value in diagnosing anticholinesterase poisoning in a marine fish.

The acetylcholinesterase (AChE)-inhibiting insecticides are now produced in greater quantities than the chlorinated hydrocarbon insecticides (Fowler and Mahan, 1971; Lawless *et al.*, 1972) and may be an increasing threat to the estuarine environment. More than 100×10^6 kg of AChE-inhibiting insecticides are produced annually in the United States (Fowler and Mahan, 1971; Lawless *et al.*, 1972) and they may now occur more frequently in coastal waters because of increased municipal, industrial, and agricultural usage. A possible threat exists from direct spray of estuaries for mosquito control (Coppage and Duke, 1971). Short-term lethal concentrations in water to aquatic organisms typically range from a few ng/liter to several mg/liter (Butler, 1963, 1965; Cope, 1963, 1965; Sanders and Cope, 1966; Pimentel, 1971) and, even though monitoring for residues of the AChE-inhibiting organophosphates has been rare, a recent study in Texas (Dupuy and Schulze, 1972) showed that organophosphate insecticide residues were not uncommon in coastal rivers. As many as five organophosphates have been found in a pooled sample of a marine fish, with a total combined residue of 0.624 ppm (P. A. Butler, unpublished observation).

The mode of action of organophosphate insecticides in vertebrates is generally regarded as disruption of nerve impulse transmission in the central and peripheral

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nervous systems by inhibition of AChE, which is the enzyme that modulates the amounts of the neurotransmitter acetylcholine (O'Brien, 1960, 1967; Heath, 1961; Koelle, 1963; Ehrenpreis, 1967; Karczmar, 1970; Aldridge, 1971). The actual toxic agents are metabolites of the insecticides bound to *O*-serine in the esteratic site of AChE (O'Brien, 1960; Koelle, 1963; Karczmar, 1970). No practical means of extracting the enzyme-bound compounds from environmental samples of organisms has been developed, but inhibition of AChE by the compounds can be measured. Measurement of normal and inhibited activity of this enzyme in aquatic animals not only offers a means of detecting serious pollution by anticholinesterase agents, but it has the potential for indicating extent of poisoning of animals in the actual environment.

Recent laboratory and field studies have indicated that brain-AChE inhibition in fishes is related to organophosphate insecticide poisoning. Fish injected with organophosphate insecticides show brain-AChE inhibition (Murphy *et al.*, 1968). A specific level of brain-AChE inhibition was shown to be related to deaths that occurred in a test population of sheepshead minnows (*Cyprinodon variegatus*) exposed to organophosphate insecticides in water under controlled static conditions in the laboratory (Coppage, 1972). Similar findings were made for AChE inhibition in brains of cod (*Gadus callarias*) exposed in seawater in the laboratory to paraoxon, a metabolite of the organophosphate insecticide parathion (Alsen *et al.*, 1973). Under ambient flowing seawater conditions, four species of estuarine and marine fishes had relatively consistent levels of brain AChE inhibition when deaths occurred from organophosphate pesticide exposure (Coppage and Matthews, 1974). Also, several field studies have shown that AChE inhibition in fish brain is correlated with water pollution or spraying with organophosphate pesticides in both fresh and estuarine water (Williams and Sova, 1966; Holland *et al.*, 1967; Mayer and Walsh, 1970; Carter, 1971; Macek *et al.*, 1972). A field study of three species of estuarine fishes showed that brain-AChE inhibition was correlated with mosquito control operations with the organophosphate malathion (Coppage and Duke, 1971).

More information on the relationship of AChE inhibition to poisoning and deaths of estuarine animals is needed to diagnose poisoning by anticholinesterase agents in the aquatic environment. In this report, we define levels of reduction of AChE activity that are critical in short-term lethal exposures of an estuarine and marine fish, *Lagodon rhomboides* (pinfish), to the organophosphate insecticide naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate).

Although studies have not been made on the persistence of anticholinesterase transformation products of naled in seawater and fishes, its application to water could possibly produce toxic effects. For example, about 21,819 kg of naled were sprayed aerially in a single coastal mosquito control operation covering 241,317 hectares (Pinkovsky, 1972). This quantity of naled would give a theoretical concentration of about 30 μg/liter in a water depth of 30 cm/hectare of water surface sprayed. Naled is acutely toxic to aquatic animals when added to water in quantities ranging from less than 1 μg to several hundred μg/liter of water (Butler, 1963, 1965; Cope, 1963, 1965; Sanders and Cope, 1966). Naled is not regarded as a "persistent" pesticide but short-term exposure to "nonpersistent" organophosphate pesticides may produce death or persistent effects. For example, malathion is not regarded as a "persistent" pesticide, but it has caused inhibition of AChE in fish brains for 30 days after only 12

hr exposure in the laboratory to a nominal concentration of 100 μg/liter (Weiss, 1961). Brain-AChE of a fish species in an estuary sprayed with malathion was inhibited and remained inhibited 50 days after spraying was discontinued (Coppage and Duke, 1971). Relatively irreversible AChE inhibition apparently occurs.

METHODS

The AChE of the pinfish brain was characterized and assayed with a pH-stat method previously described (Coppage, 1971). Normal brain AChE activity was determined with pinfish (65–125 mm total length) taken randomly from wild populations over a 2-year period. Normal AChE activity of 31 samples (each sample was an homogenate of pooled brains taken from five pinfish) had a coefficient of variation of only ±9% for the period. Each assay sample for AChE inhibition consisted of pooled brains taken from 4–6 fish that survived naled exposure for a designated time, and percentage inhibition was determined by comparison with mean normal activity. Dead fish were not used to interpret AChE inhibition because it cannot be applied in practical field studies where it is not known how long fish have been dead and subject to loss of AChE activity due to protein destruction.

In each test, 4–12 replicates of 10 fish each were exposed to technical grade pesticide in 8-liter acrylic plastic aquaria that received a mixture of flowing seawater (400 ml/min) and naled from a common source. The naled was dissolved in benzene and infused into seawater by means of a syringe pump. Solvent infusion never exceeded 2.5 mg/liter of water. Benzene did not significantly affect AChE activity of fish exposed to 8 mg/liter for 72 hr (Table 1). Pesticide concentration in the water was expressed as μg added per

TABLE 1

BRAIN-ACETYLCHOLINESTERASE ACTIVITY IN CONTROL PINFISH AND POPULATIONS SUBJECTED TO SUBLETHAL AND LETHAL EXPOSURE TO NALED

Nominal concentration (μg/liter)	Hours exposed	Percent killed	Number of samples	Mean AChE activity[a]	SD	Percent less AChE activity than control
Control	—	—	31	2.03	0.18	—
8000 benzene only	72	0	3	2.16[b]	0.10	—
15	24	0	4	0.84[c]	0.11	59
15	48	0	4	0.77[c]	0.19	62
15	72	0	4	0.72[c]	0.10	65
15	96	0	4	0.60[c]	0.13	70
25	72	40–60	4	0.23[d]	0.03	89
55	48	40–60	4	0.33[d]	0.10	84
75	24	40–60	3	0.24[d]	0.00	88

[a] Expressed as μmol of acetylcholine hydrolyzed/hr/mg brain tissue.
[b] Not significantly different from control.
[c] Significantly less activity than controls ($p < 0.001$).
[d] Significantly less activity than fish exposed to 15 μg/liter for same period ($p < 0.01$).

liter. No attempt was made to chemically analyze naled or its transformation products in the water. Temperature range was 18–23°C, and salinity was 23–29 parts per thousand during the tests.

To determine the extent of AChE inhibition resulting from a near-median kill we assayed the survivors of test in which 40–60% of the test populations were killed by exposure to naled in 24, 48, and 72 hr. Brain-AChE activity was measured at 24 hr for the 24-hr lethal exposure (75 μg naled/liter), 24 and 48 hr for the 48-hr lethal exposure (55 μg naled/liter), and at 24, 48, and 72 hr for the 72-hr lethal exposure (25 μg naled/liter). This was accomplished by exposing several groups of fish, in separate aquaria, to the same source of naled in flowing seawater. At each specified time interval, 3–4 replicate groups of 4–6 fish each were taken from the replicate aquaria and their brain-AChE was measured. Also, we exposed other groups of pinfish for 96 hr in water to which 15 μg naled/liter were added and measured their brain-AChE activities at 24, 48, 72, and 96 hr.

RESULTS AND DISCUSSION

Inhibition data for fish, expressed as percentage reduction of AChE activity when compared with mean normal activity, are summarized in Fig. 1. Statistical comparisons of AChE activities of fish exposed to lethal concentrations were made with fish exposed to the sublethal concentration (Student's t test, $p < 0.01$) (Table 1).

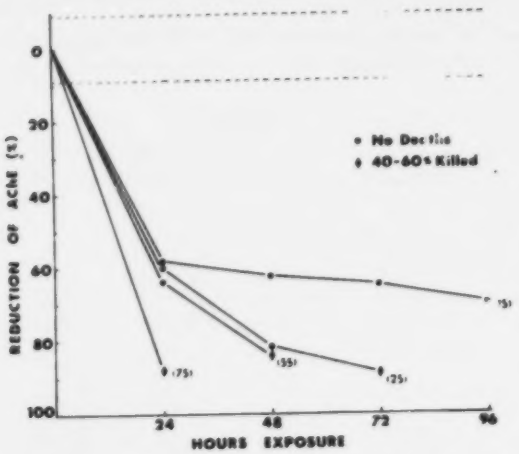

FIG. 1. Inhibition of brain-acetylcholinesterase activity by sublethal and lethal exposure to naled. Each experimental point represents the mean of 3–4 replicate tests. The amounts of naled added to the seawater, in μg/liter, for a particular test or test sequence are given in parentheses. One SD from normal during a 2-year sampling of wild fish populations is indicated by dashed lines.

For the same length of exposure, lethal concentrations of naled always produced a significantly greater inhibition of brain-AChE than sublethal concentrations (Table 1). Mean reductions of AChE activity in all lethal exposures that killed 40–60% of the test populations were similar (84–89%), regardless of concentration of naled or length

of exposure. The mean inhibition caused by 15 μg/liter (sublethal) was greater in each succeeding 24-hr period reaching a maximal inhibition of 65% in 72 hr and 70% in 96 hr. Thus, in exposures of up to 72 hr, brain-AChE inhibition in excess of 83% indicates a high probability of impending death in an exposed population. In the "sublethal" exposure the increasing inhibition with time and the mean reduction of 70% at 96 hr suggest that, if exposure continued, a lethal level of AChE inhibition may occur. These findings strongly support earlier findings for other fish species (Coppage, 1972; Alsen *et al.*, 1973; Coppage and Matthews, 1974) that brain-AChE inhibitions of about 70–80% are critical in short-term lethal poisoning by organophosphate insecticides. The relatively specific levels of AChE inhibition during "kills" show that it is unnecessary to rely on the dubious interpretation of residues alone to determine poisoning and cause of "kills" in the environment. Correlation of AChE inhibition with insecticide usage or residue analysis should be sufficient to establish identity of the compound or compounds causing poisoning and "kills" of fishes in aquatic systems.

The large AChE inhibitions (59–65%) caused by "sublethal" exposure indicate pollution can be readily detected, possibly before acute poisoning occurs. However, even if acute lethal poisoning does not occur during organophosphate insecticide exposure, depression of AChE in vertebrates may cause physiological and behavioral modifications (Koelle, 1963; Karczmar *et al.*, 1970) that reduce animal survival ability. Cumulative reduction of AChE by repetitive exposure to some organophosphate pesticides has been demonstrated in some vertebrates (Heath, 1961; Koelle, 1963; Karczmar, 1970), and this possibly happens in fish repetitively exposed in the environment. For example, repetitive exposures of fish to short-term "sublethal" concentrations of azinphosmethyl has resulted in deaths (Lahav and Sarig, 1969). This may be of significance because anticholinesterase mosquito control chemicals are often applied to marshes several times a month.

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Marked DDE Impairment of Mallard Reproduction in Controlled Studies

by

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DDE in concentrations of 10 and 40 p.p.m. in the feed of penned mallards induced significant eggshell thinning and cracking and a marked increase in embryo mortality. DDD and DDT impaired reproduction, but less severely than DDE, a widespread breakdown product of DDT.

CORRELATIONS have been found between drastic declines in populations of raptors and fish-eating birds, unusually thin eggshells in such populations, and environmental exposure to organochlorine pesticides, particularly DDT [1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane] and DDE [1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene][1-3]. DDE has been prevalent in wild ducks: it was found in the wings of 900 mallards (*Anas platyrhynchos*) and black ducks (*Anas rubripes*) monitored in 1964 (ref. 4), in tissues and organs of 104 mallards and lesser scaup (*Aythya affinis*)[5], and in eggs of Atlantic flyway black ducks which have been reproducing more slowly than usual since 1955 (ref. 6).

DDE, while widely distributed throughout the environment as a stable metabolite of DDT, is not produced commercially and not readily obtainable. Controlled studies of the effects of DDT on avian reproduction are well documented, but we know of only one such investigation of DDE[7].

In 1965, 2 year studies of the effects of organochlorines on mallard reproduction began at this centre. DDT was investigated in 1965–66 and DDE and DDD [1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethane] in 1967–68. We used *p,p'*-isomers of DDT and DDE and a technical formulation of DDD (64 per cent *p,p'*-isomer, 20 per cent *o,p'*-isomer, 16 per cent impurities). Test mallards had been hatched in an incubator, the progeny of wing-clipped breeders phenotypically indistinguishable from wild birds. During the investigation birds were kept in wooden pens on concrete slabs supplied with running water and spread with straw. A completely randomized design was used throughout. Five or six hens and two drakes were allotted to each pen, and each treatment was replicated in three to five pens.

Pesticides were administered in diets. DDE and DDD were fed in concentrations of 10 or 40 p.p.m.; DDT was tested in concentrations of 2·5, 10 or 40 p.p.m. reduced to 25 p.p.m. after breeders had died. We chose 10 p.p.m. as a residue easily encountered in natural foods, and 40 p.p.m. as approaching the largest sustained concentration not lethal to breeders. Concentrations are expressed as p.p.m. of toxicant in dry feed: wet weight concentrations, as in natural diets, would be about one-third the dry weight concentrations. Diets were prepared by dissolving the pesticides in corn oil and mixing one part of oil solution with ninety-nine parts of commercial duck feed.

Birds received treated diets freely from several weeks before the onset of the first laying season until the second year of laying. Eggs were collected daily for 2 months during each season and stored at 16° C and 55 per cent relative humidity. At fortnightly intervals they were examined for cracked shells, incubated, and candled after 2 and 3 weeks to measure embryonation and embryo survival. Hatchlings were banded on the leg for identification with the parental pen and observed on diet free of pesticide for 14 days. Productivity data were recorded for each pen for every 2 week set of eggs (Table 1*a*). From these data we derived the parameters of reproductive success presented in Table 1*b*, arranged sequentially to reveal stages of development affected by treatments.

Eggs with sound shells from alternate 2 week sets were sampled to measure shell thickness and density. The thickness of dried shell plus membrane at the waist was measured with a micrometer calibrated in 0·01 mm units. Limited measurements of relative shell strength were made using a technique[8] in which beta particles are bounced from the shell and backscatter is counted by a spectrometer. The amount of backscatter is related to both shell density and thickness.

All estimates of reproductive success were analysed by weighted analysis of variance; pen statistics were weighted according to the numbers of eggs or ducklings on which they were based. Angular transformations were applied to percentages for analysis[9]; means were separated by the methods of Duncan[10] and Kramer[11].

DDE in concentrations of 10 and 40 p.p.m. severely impaired reproductive success ($P < 0.01$). The effects of both concentrations were similar except that they were initiated more rapidly by the larger concentration. DDE evidently affected eggshell formation, so that shells were 13 per cent thinner than normal (Table 2). Thin shells cracked readily; cracking, detectable after 1 month of dosage, reached 25 per cent (Table 1*b*). Shell fractures varied from hairline cracks to obvious breaks. Eggs with hairline cracks hatched at about half the rate of whole eggs; those with definite fractures did not hatch.

DDE caused reduced hatchability of eggs with uncracked shells (Table 1*b*). Embryo mortality during the final (fourth) week of incubation varied from 30 to 50 per cent higher than expected, even though survival during the first 3 weeks was normal. The analysis also suggests lower embryonation. The survival of hatchlings to 14 days was not significantly affected. Summary parameters "percentage of 14 day ducklings of embryonated eggs" and "14 day ducklings per hen" showed that the success of embryonated DDE eggs was from one-third to one-half that of control eggs, and duckling production per hen was reduced as much as 75 per cent.

DDD did not cause demonstrable changes in shells. It impaired reproductive success ($P < 0.05$) but less severely than did DDE. Impairment was cumulative and not precipitous.

DDT induced significant ($P = 0.05$) thinning of shells only when in a concentration of 25 p.p.m., which resulted in 18 per cent cracking. But DDT metabolizes to DDE,

Table 1a. REPRODUCTIVITY DATA FROM PENNED MALLARDS MAINTAINED FOR TWO SEASONS ON FEED CONTAINING DDE, DDD OR DDT

	Season	None	DDE 10	DDE 40	DDD 10	DDD 40	Season	None	DDT 2·5	DDT 10	DDT 40–25*
		Pesticide added to feed (p.p.m.)									
Pens of birds	1967	4	4	4	3	4	1965	5	5	5	5
	1968	4	4	3	3	4	1966	4	4	5	4
Hen-seasons (8 weeks each)	1967	20	20	20	14·6	20	1965	20	20·0	20·6	20·7
	1968	17	18	8	11	18	1966	19	20·5	20·4	14·1
Eggs laid	1967	606	620	602	484	539	1965		(data incomplete)		
	1968	656	664	317	331	490	1966	319	404	665	341
Eggs cracked	1967	34	61	117	35	32	1965		(data incomplete)		
	1968	31	159	79	24	35	1966	13	26	55	60
Eggs incubated	1967	626	530	459	424	477	1965	489	563	563	460
	1968	607	485	228	291	436	1966	296	355	578	246
Eggs embryonated	1967	507	399	320	313	352	1965	408	397	393	231
	1968	543	261	154	275	302	1966	199	310	446	180
Live 3 week embryos	1967	492	384	276	299	325	1965	374	372	357	204
	1968	518	232	128	266	280	1966	180	287	388	124
Normal hatchlings	1967	357	205	131	150	185	1965	253	226	197	112
	1968	318	91	38	111	143	1966	135	192	248	85
14 day old ducklings	1967	327	167	113	136	166	1965	210	203	183	74
	1968	274	69	26	94	128	1966	115	169	209	44

* Dose of 40 p.p.m. reduced to 25 p.p.m. after first season.

Table 1b. SEQUENTIAL PARAMETERS OF REPRODUCTIVE SUCCESS AMONG PENNED MALLARDS MAINTAINED THROUGH TWO SEASONS ON FEED CONTAINING DDE, DDD OR DDT

Parameter	Season	None	DDE 10	DDE 40	DDD 10	DDD 40	Season	None	DDT 2·5	DDT 10	DDT 40–25*
		Chemical and p.p.m. added to feed									
Eggs laid per hen in 8 weeks	1967	34·8	31·0	30·1	33·2	27·0	1965		(data incomplete)		
	1968	39·2	36·9	39·6	30·1	27·2	1966	16·8	19·7	23·4	24·2
Per cent cracked eggs	1967	5	10†	19‡	7	6	1965		(data incomplete)		
	1968	5	24‡	25‡	7	7	1966	4	6	8	18†
Per cent embryonated of eggs incubated	1967	81	75	70	74	74	1965	83	71	70	50
	1968	89	54†	68	95	69	1966	70	87	77	65
Per cent embryos alive at 3 weeks' incubation	1967	97	96	86	96	92	1965	92	94	91	86
	1968	95	89	83	97	93	1966	90	93	87	78
Per cent normal hatchlings of 3 week embryos	1967	73	53†	47†	50†	57	1965	68	61	55	55
	1968	61	39†	30†	42	51	1966	75	67	64	69
Per cent normal hatchlings alive at 14 days	1967	92	81	86	91	90	1965	83	90	93	86†
	1968	86	76	68	85	90	1966	85	88	84	82‡
Per cent 14 day ducklings of embryonated eggs	1967	64	42‡	35‡	43†	47†	1965	51	51	47	32
	1968	50	26†	17‡	34	42	1966	58	55	47	28‡
14 day ducklings per hen §	1967	16·4	8·4†	5·7‡	9·3	8·3†	1965	7·0	6·8	6·2	2·5
	1968	16·1	3·8‡	3·3‡	8·6†	7·1†	1966	6·0	8·2	7·4	3·1

Statistics are based on the data in Table 1a.

* Dose of 40 p.p.m. reduced to 25 p.p.m. after first season.

† Difference from control statistically significant ($P < 0.05$).

‡ Difference from control highly significant ($P < 0.01$).

§ Ratios unadjusted for 5 per cent of sound eggs per treatment removed for residue analysis and shell measurement.

Table 2. THICKNESS OF EGGSHELLS OF PENNED MALLARDS MAINTAINED THROUGH TWO SEASONS ON FEED CONTAINING DDE, DDD OR DDT

	Season	None	DDE 10	DDE 40	DDD 10	DDD 40	Season	None	DDT 2·5	DDT 10	DDT 40–25*
		Pesticide added to feed (p.p.m.)									
No. of eggs	1967	20	25	23	21	26	1965		(shells not measured)		
	1968	28	21	10	16	19	1966	12	12	12	12
Shell thickness (mm)	1967	0·38	0·35	0·33†	0·37	0·37	1965	—	—	—	—
	1968	0·37	0·33†	0·32*	0·35	0·35	1966	0·38	0·36	0·35	0·33†

* Dose of 40 p.p.m. reduced to 25 p.p.m. after first season.

† Difference from control highly significant ($P < 0.01$).

and so stored metabolic DDE may have induced thinning. DDT in a concentration of 25 p.p.m. also reduced duckling survival ($P < 0.01$) approximately 35 per cent. The 50 per cent deficit in ducklings per hen approaches significance but fails because of variation in pen fertility rates. DDT in concentrations of 2·5 and 10 p.p.m. did not have measurable effects on reproduction. None of the treatments induced crippling among hatchlings.

Only eggs saved for residue analysis were available for beta backscatter counts. Shells produced by birds receiving DDE and 25 p.p.m. of DDT yielded counts 13 per cent below those of control eggs, the same percentage difference found in measurements of shell thickness.

We thank Mr E. Cromartie and Dr R. M. Prouty for DDE, Mr C. Vance for carrying out aviculture and candling, Mrs H. M. Nelson for computations, Mr P. E. James for a beta backscatter facility and Mr E. J. Koch for statistical consultation.

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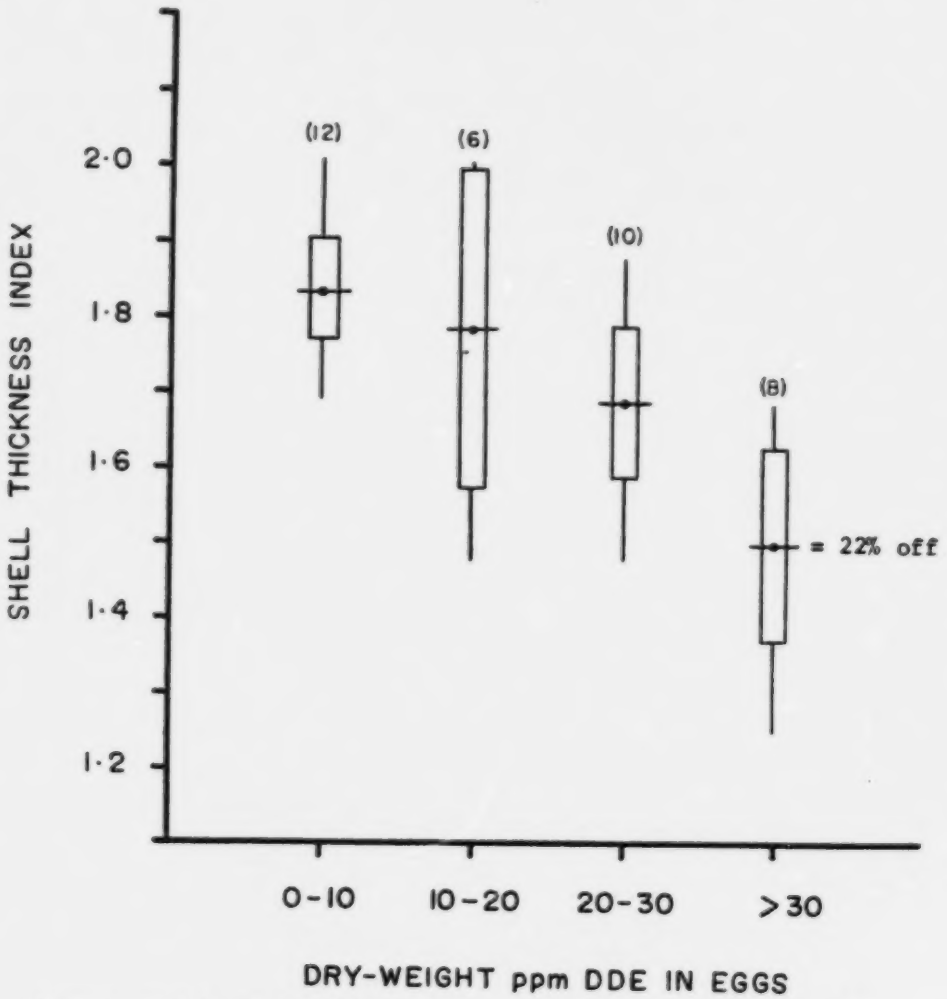

FIGURE 3. Variation of shell-thickness index with DDE concentration in Prairie Falcon eggs. Sample numbers are in brackets, horizontal lines are means, boxes are 95 percent confidence limits, and vertical lines are sample ranges.

fed to the falcons immediately before egg laying. Prairie Falcons in western Canada return to the nesting territories several weeks before egg laying and so much of the insecticide residues in the eggs may be accumulated from the prey in the vicinity of the nest during this period.

Individual variation in residues in the eggs within any one area probably results from specific prey abundance or food preferences of individual birds. Considerable individual differences in food preferences are already established

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SIGNIFICANCE OF CHLORINATED HYDROCARBON RESIDUES TO BREEDING PELICANS AND CORMORANTS

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INTRODUCTION

THIS PAPER reports levels of chlorinated hydrocarbons present in eggs and spring food of Double-crested Cormorants (*Phalacrocorax auritus*) and White Pelicans (*Pelecanus erythrorhynchos*) and the effects the residues might have upon the reproductive physiology of these species. On the basis of research concerning fat-kinetics in certain migratory birds (Hanson 1962, Weise 1963, Brenner 1967, and others), there is good reason to believe that residues in the eggs provide a reliable index to fat-stored contamination in the female, especially in birds with small clutches. The precise relationships have yet to be studied in detail (Lockie 1967, Stickel 1968).

FIGURE 3. Relationships between DDE residues (A), estimated PCB residues (B), and shell thickness in Double-crested Cormorants, plotted on a colony-basis. Individual points are numbered in accordance with Figure 1. Open circles represent renest colonies (the original colony of the season was destroyed or disturbed away from the first-nest site, therefore, phenologically behind other colonies from the same general latitude and longitude) and closed circles represent first-nest colonies. "MU" in the upper figure represents the museum mean thickness (Table 5), bounded by 95% Confidence Limits. Figure 3A, $P<0.001$; Figure 3B, $P<0.01$. The line-of-fit for renests in A. was fitted by eye but clearly resembled the calculated regression based on individual pools. A line-of-fit for renests in B., though significant on an individual-pool basis, was not obvious on a colony basis.

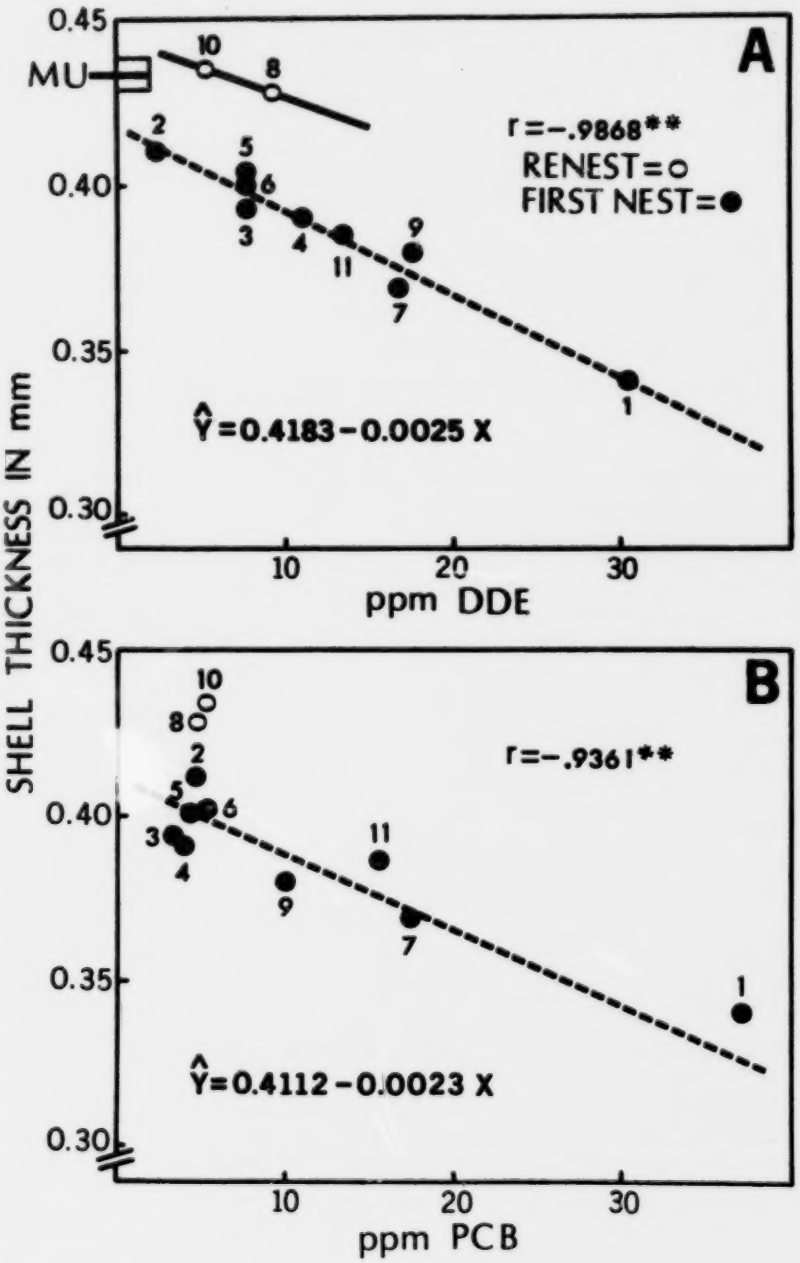

Figure 3.

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Food and Drug Administration

Urinary Excretion of Insecticide Metabolites

Excretion of Para-nitrophenol and DDA as Indicators of Exposure to Parathion and DDT

Homer R. Wolfe, Wenatchee, Wash; William F. Durham, PhD, Perrine, Fla; and John F. Armstrong, Wenatchee, Wash

The urinary excretion of *p*-nitrophenol and of dichlorodiphenylacetic acid (DDA) was measured in spraymen applying formulations containing both parathion and DDT. In each instance, excretory level of metabolite correlated well with exposure to the pesticide. Para-nitrophenol excretion levels showed changes of greater magnitude in response to a specific exposure than did DDA excretion. However, in general the excretory patterns for *p*-nitrophenol and DDA were similar. Both metabolites reached peak levels at about the same time interval (*p*-nitrophenol, 8.7 hours; DDA, 10.1 hours) after exposure began. Excretion of both *p*-nitrophenol and DDA tended to increase with an increase in ambient temperature. Excretion curves for both metabolites showed diurnal variation during the period after exposure had ended. On the average for a specific exposure, more parathion-derived material than DDT-derived material was recovered in the urine.

EXCRETION of *p*-nitrophenol, a metabolite of the organic phosphorus pesticides parathion, methyl parathion, EPN, and dicapthon, is a good indicator of exposure to these compounds.[1,2] No normal urine constituents and few metabolites of common drugs seem to react in the colorimetric test for *p*-nitrophenol.[3] Excretion levels of *p*-nitrophenol rise promptly in response to parathion exposure and return to the nondetectable level after several days, as has been noted by W. F. Durham, H. R. Wolfe, and J. W. Elliot (unpublished data). Para-nitrophenol excretion is a more sensitive measure of exposure to parathion than is blood cholinesterase level[1] though the latter seems to correlate better with occurrence of poisoning than does *p*-nitrophenol excretion.[1] The excretion technique has been used to determine exposure to parathion of persons with occupational or environmental exposure[1,2,4] and of poisoning cases.[1,2,5,6]

Dichlorodiphenylacetic acid (DDA) excretion correlates reasonably well with DDT storage in body fat.[7] DDA excretion has been used to measure DDT exposure level in pesticide applicators,[7] formulating plant workers,[8] and volunteers given daily known oral doses of DDT for periods up to two years.[9] Similar findings have been noted by W. J. Hayes, Jr., W. E. Dale, and C. I.

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Parts of this paper were presented at the fifth annual meeting of the Society of Toxicology, Williamsburg, Va, March 15, 1966.

Reprint requests to Wenatchee Research Section, Food and Drug Administration, PO Box 73, Wenatchee, Wash 98801 (Mr. Wolfe).

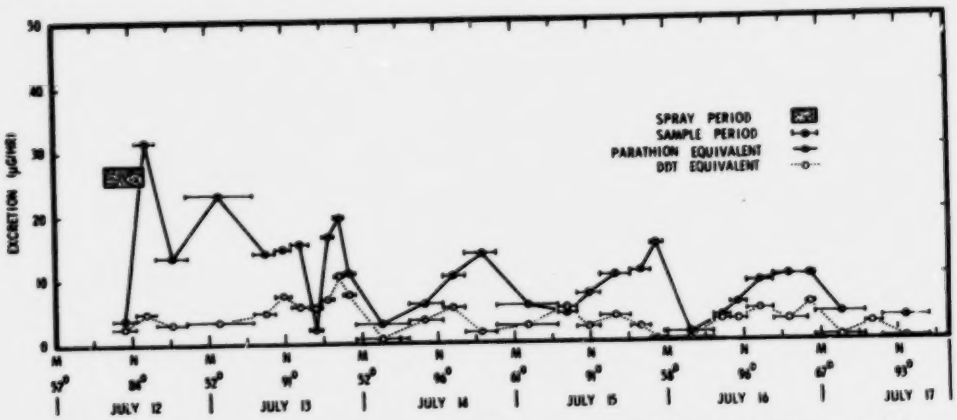

Fig 1.—Excretion of p-nitrophenol and DDA by a sprayman following a single exposure period. Temperatures indicated are daily maximum and minimum. Spray period represents the time of actual exposure. Sample period represents the interval covered by collection of a voiding of urine (total number of hours per voided specimen). M indicates midnight; N, noon.

Pirkle (unpublished data). DDA excretion levels have generally been too near the lower limit of detectability, when using methods available at the time of this study, to be useful for study of the general population or persons with environmental exposure only.[7] No reports have been found on studies of short-term variations in DDA excretion level in spraymen following ordinary brief exposure.

This study investigated the usefulness of DDA excretion as a method of measuring acute exposure of orchard spraymen to DDT. It also compared excretory patterns of the metabolites, DDA and *p*-nitrophenol, in spraymen exposed to formulations containing DDT and parathion.

Materials and Methods

DDA and *p*-nitrophenol excretion studies were carried out during a three-year period and included 18 different exposure situations involving 12 spraymen. In 14 exposure situations, the subjects applied formulations containing both DDT and parathion. In the other four instances, DDT alone was applied. Formulations were prepared generally by mixing 2 lb of 50% wettable DDT powder and 1 lb of 25% wettable parathion powder in 100 gal of water. The resulting spray mixture contained about 0.12% DDT and 0.03% parathion. Formulations which contained DDT alone were prepared with the same amount of DDT as the mixed formulations. The spray was applied to apple or pear orchards at the rate of 600 to 1,000 gal per acre using air-blast spray machines: dosage rates were 12 to 20 lb per acre for parathion and 48 to 80 lb per acre for DDT.

Subjects collected 24-hour specimens of urine for several days during and following their spray exposure periods. Each voiding was kept in a separate container and time of excretion was recorded. Samples were analyzed for *p*-nitrophenol content by the method of Elliott et al[9] and for DDA by the method of Cueto et al.[10] Para-nitrophenol was expressed as parathion equivalent (actual *p*-nitrophenol × 2.09), and DDA as DDT equivalent (actual DDA × 1.24). Results for both metabolites were calculated as amount excreted per hour, rather than on a parts per million basis, to minimize the dilution factor. The total amount of each pesticide excreted subsequent to each exposure was also calculated.

A total of 386 urine samples was analyzed for DDA and *p*-nitrophenol; 77 additional samples were tested for DDA alone.

Results and Comment

Total excretion of *p*-nitrophenol and DDA by the 12 spraymen studied is summarized in the Table; excretion of *p*-nitrophenol and DDA by three workers representative of this group is shown in Fig 1 to 3. Individual spraymen in Fig 1 and 2 had single exposures; the individual in Fig 3 had three different exposures.

Para-nitrophenol excretion levels correlated well with exposure to parathion and increased rapidly after spraying, then de-

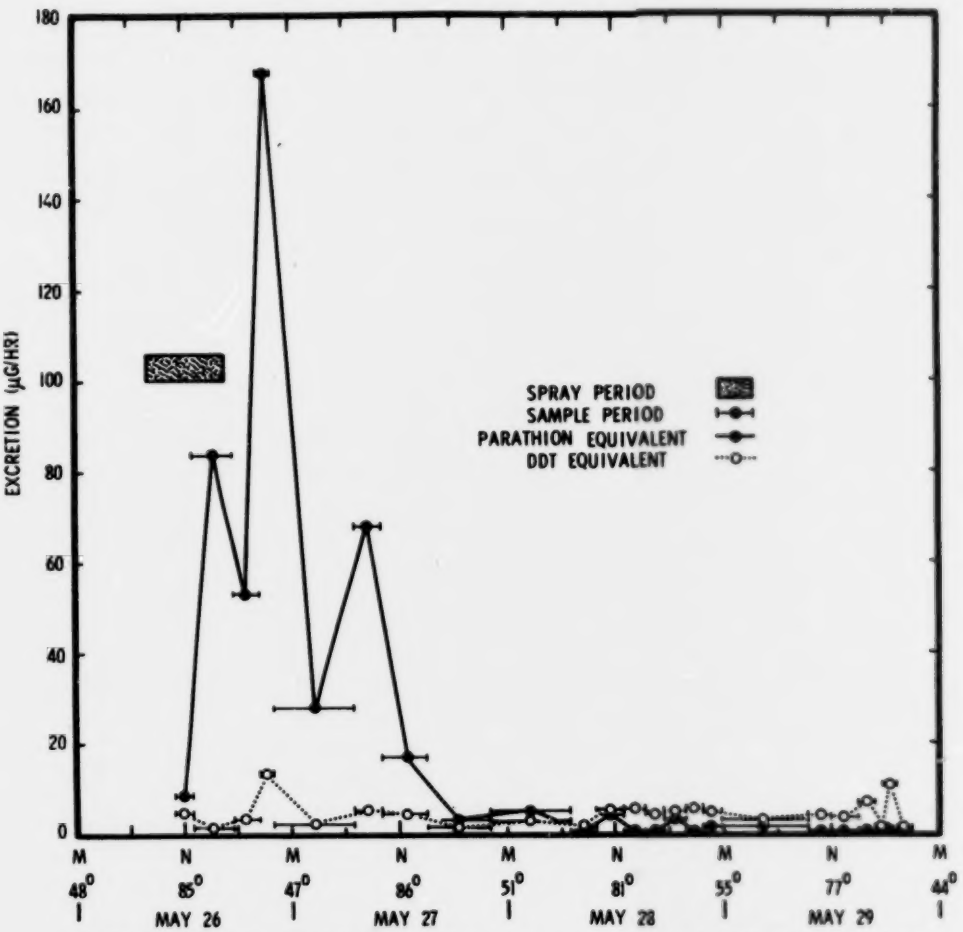

Fig 2.—Excretion of p-nitrophenol and DDA by a sprayman following a single exposure period. Temperatures indicated are daily maximum and minimum. Spray period represents the time of actual exposure. Sample period represents the interval covered by collection of a voiding of urine (total number of hours per voided specimen). M indicates midnight; N, noon.

creased promptly. The average peak excretion level occurred 8.7 hours after exposure began. There was often a diurnal variation in excretion pattern on days subsequent to the day of exposure, illustrated well in Fig 1. Para-nitrophenol levels were low late at night and early in the morning, reaching higher levels during the afternoon and evening. Excretion became insignificant five to eight days after exposure. Differences in chronicity of excretion are illustrated by comparing Fig 1 with Fig 2. In the former, measurable excretion lasted for five days; in the latter, levels were insignificant two days after exposure. The sprayman whose *p*-nitrophenol excretion is shown in Fig 2 excreted significantly higher levels of this metabolite in the early hours after cessation of exposure compared with the subject in Fig 1. These high excretion levels may represent early complete metabolism and clearance of the poison by the subject in Fig 2 while more sustained excretion levels may be associated with lesser capacity to metabolize the poison. However, in all the subjects studied following multiple daily exposure there was little or no indication of progressive buildup of excretion levels from day to day (Fig 3).

In each exposure situation to DDA which was studied, subjects showed detectable ex-

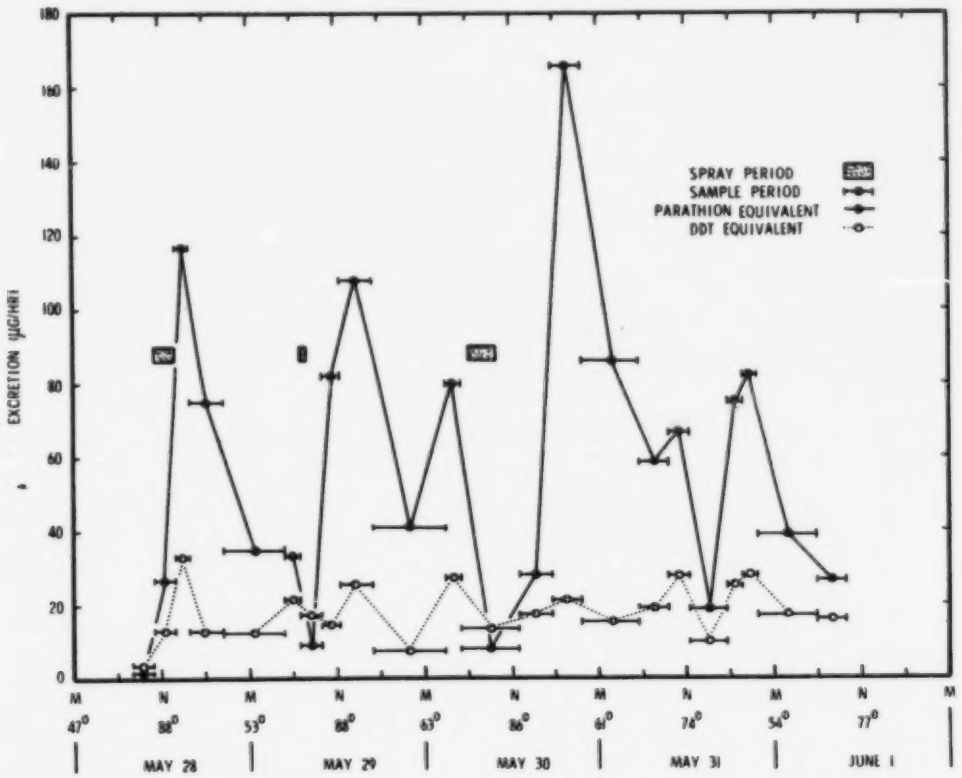

Fig 3.—Excretion of p-nitrophenol and DDA by a sprayman following three exposure periods. Temperatures indicated are daily maximum and minimum. Spray period represents the time of actual exposure. Sample period represents the interval covered by collection of a voiding of urine (total number of hours per voided specimen). M indicates midnight; N, noon.

Excretion of Para-nitrophenol and DDA by Orchard Spraymen During and Following Exposure to DDT and Parathion

Sprayman	Excretion Period (Days)	Total Excretion (mg)	
		Para-nitrophenol	DDA
1	8.5	4.2	3.4
2	4.2	5.1	1.6
3	3.7	1.5	1.1
4	6.0	3.8	2.3
5	4.5	1.4	0.3
6	5.7	1.2	0.4
7	4.8	2.2	0.9
8	4.0	...*	0.1
9	4.0	...*	0.4
10	4.5	...*	0.2
11	4.5	...*	0.5
12	3.7	...*	0.2
Mean	4.83	2.78	0.95

* Exposed to DDT only.

cretion levels of DDA ranging from 2µg to 11µg per hour (calculated at DDT equivalent) during the prespray period. This finding for spraymen contrasted with that for the general population in which 74% of the subjects had nondetectable levels of DDA excretion.[7] It indicates the generally high DDT exposure level of spraymen.

Following each DDT exposure, the excretory level of DDA increased, reaching a maximum level, on the average, 10.1 hours after spraying began. In a few instances, particularly where spraying was prolonged, the highest excretion occurred soon after or even before exposure was terminated. Following the postexposure peak, DDA excretion generally decreased rapidly to, or near, the preexposure level. On days subsequent to the day of actual exposure, excretion tended to show a diurnal pattern. DDA excretion continued at a low but detectable level during the entire sampling period for most spraymen.

Excretory patterns for *p*-nitrophenol and DDA were similar qualitatively. Both metabolites in most cases reached peak levels at about the same time. The mean interval between beginning of spraying and peak excretion was 8.7 hours for *p*-nitrophenol and 10.1 hours for DDA.

DDA excretion levels showed changes of lesser magnitude in response to a specific exposure than did *p*-nitrophenol levels. Urinary *p*-nitrophenol concentrations tended to be higher than DDA levels during the exposure and early postexposure periods. The mean maximum excretion rate for DDA was 22.5μg/hr with a range of 11μg to 44μg per hour. The corresponding average value for *p*-nitrophenol was 79.2μg/hr with a range of 30μg to 168μg per hour. In one exception, not included in this calculation (since it was suspected that accidental oral ingestion of the spray formulation occurred), the DDA excretion rate reached 229μg/hr and the *p*-nitrophenol excretion reached 561μg/hr at the same time. Generally, *p*-nitrophenol excretion fell to a nondetectable level by the fifth to eighth day after exposure while DDA excretion continued to be measurable throughout the sampling period.

The excretion of *p*-nitrophenol and DDA increased with increase in ambient temperature. Excretion curves for both compounds showed diurnal variation after exposure had ended.

In spraymen exposed to both parathion and DDT, the total amount of *p*-nitrophenol excreted per exposure was 1.24 to 4.43 times (mean, 2.56 times) greater than that for DDA. Since the original formulation contained four times as much DDT as parathion, on a comparative basis 10.2 times as much parathion as DDT was accounted for by urinary excretion of metabolites.

It is possible to calculate the relative toxic hazard of the amounts of parathion and DDT absorbed (based on total *p*-nitrophenol and DDA excreted [Table]) by the 12 spraymen in this study. The mean amount of parathion absorbed was 2.78 mg or about 0.04 mg/kg. Assuming that this absorption was predominantly via the skin and that man and rat are equally susceptible, this amount corresponds to only about 0.02% of the lethal dose level for 50% survival of the group (LD_{50}). Because the exposure and absorption took place over a period of several days (Table), this low level is even less significant in terms of acute hazard. Mean DDT absorption was 0.95 mg or about 0.02 mg/kg, corresponding to about 0.0008% of the LD_{50} level. However, the actual difference in fraction of toxic dose absorbed may be less than these figures show since some unknown fraction of absorbed DDT is stored in body fat and an additional fraction is excreted via the bile into the feces. Nevertheless, the calculated difference in fraction of toxic dose absorbed correlates well with the known greater occupational hazard of parathion compared to DDT.

In view of the dissimilar patterns of metabolism for DDT and parathion, it is surprising that DDA and *p*-nitrophenol show such similar excretion patterns. That peak excretion levels for both metabolites occur at about the same time was particularly unexpected. In the metabolic process, parathion is oxidized to para-oxon by enzymes in the liver microsomes and elsewhere.[11] Some fraction of para-oxon then reacts with cholinesterase to form a phosphorylated enzyme and free *p*-nitrophenol.[12] Another part of the para-oxon is enzymatically hydrolyzed into *p*-nitrophenol and diethylphosphoric acid.[13] More recent work indicates that the hydrolytic step may occur without parathion ever having been converted to para-oxon.[13] The outcome of exposure to parathion in a specific situation seems to depend upon the relative magnitude of cholinesterase inhibition and enzymatic hydrolysis reactions.[14]

Although metabolic reactions for DDT are less well understood, a significant fraction (which varies according to the situation) of an absorbed dose is stored in body fat.[15] Our results indicate that another fraction of the dose is converted to DDA and excreted rather promptly. Peterson and Robison[16] demonstrated in an excellent study that the metabolic pathway between DDT and DDA in the rat proceeds through DDD as an intermediate product. These authors also showed that DDE production in the rat represents a separate branch of the DDT metabolic process which does not lead to DDA. The monkey can convert DDT to DDA but does not store DDE when given DDT, although he does store DDE when fed the compound itself.[17] Our finding that DDA excretion lev-

el in spraymen increases promptly following acute spray exposure and then decreases quickly to the background level, correlates with a metabolic pathway in man through DDD rather than DDE as an intermediate. If the process proceeded through the latter route, it would be difficult to reconcile the prompt changes in DDA excretion with the known relative tendency of DDE to show increased accumulation in body fat with time.

The good correlation between excretory level and exposure in our studies indicates that excretory levels of *p*-nitrophenol and DDA can be used as indicators of the magnitude of exposure to parathion and DDT, respectively. The good correlation between excretory level for these two metabolites in individual subjects suggests that the time intervals required for metabolism of acute doses of these two unrelated pesticides are similar.

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Toxicity of Metepa to Rats

With Notes on Two Other Chemosterilants

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Although tepa, metepa, and apholate are only moderately toxic to rats by the oral route, the dermal toxicity of the first two is relatively high as compared with that of conventional insecticides. In the experiments reported, metepa caused no damage to the intestinal epithelium of rats, except following single doses in the fatal range. The organ primarily affected by repeated small doses of metepa is the testis; the ovaries and bone marrow are damaged only by higher dosage levels. Metepa at an oral dosage of 5 mg/kg/day (about 4% of the acute oral LD_{50} level daily) produced severe reduction of fertility of male rats within 22 days, sterility within 70 days, and testicular atrophy within 77 days. Half that dosage produced a smaller reduction in fertility and only partial testicular atrophy in some rats within 197 days. Dosages of 1.25 mg/kg/day or less produced no detectable effect on fertility and no histological change in the testis in 197 days. The survival of newborn rats was not affected by any dosage given to their sires.

The development and successful application of the sterile-male technique for control of the screw-worm fly, *Callitroga hominivorax*, in the south-eastern USA brought great financial benefits to livestock producers in the area. Control of the native populations of screw-worm flies was achieved by the release of large numbers of insectary-reared flies, made sexually sterile by ionizing radiation (Baumhover et al., 1955; Bushland & Hopkins, 1951; Knipling, 1959, 1960). There is interest in using this technique for the control of other insects. However, there are certain practical disadvantages inherent in insect control that depends on gamma-radiation as the sterilizing agent. These disadvantages might be overcome by the use of chemical sterilants (Knipling, 1955, 1959; Lindquist, 1961).

Smith (1963) recently reviewed the progress made in the development of chemical sterilants. From considerable laboratory investigation and some field study it appears that certain alkylating agents are the most promising chemicals for sterilization of insects. Of the alkylating agents studied to date, metepa (tris[1-2(2-methylaziridinyl)] phosphine oxide) has been the most studied and is the most promising for both flies and mosquitos. Two other analogues of ethyleneimine—tepa (tris(1-aziridinyl) phosphine oxide) and apholate (2,2.4,4,6,6-hexahydro-2,2,4,4,6,6-hexakis(1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine)—are also promising.

Wheeler (1962) published a review of the chemical and biochemical modes of action of cytotoxic alkylating agents. Hayes (1964)[3] has reviewed the toxicology of these compounds. Although they are known to produce a wide range of effects, their long-term toxicity has not been studied sufficiently to indicate which tissue or which physiological function is most sensitive to any one of them.

This paper reports on the toxicity of metepa in rats, including its effect upon reproduction when given repeatedly over a long period of time to males. The paper also reports the acute toxicity of tepa and apholate.

MATERIALS AND METHODS

The rats used in these studies were of the Sherman strain. With specified exceptions, they were individually caged, weighed at least once a week and observed daily at least five days a week for symptoms

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[3] See the article on page 721 of this issue.

of poisoning. The preparation of the rats for dermal application of the chemosterilants was similar to that described by Gaines (1960).

The compounds tested were of technical grade. Except when given to rats as a component of the diet, the compounds were dissolved in water in the appropriate concentrations for the volume of solution to be given by stomach tube at a constant rate of 5 ml per kg of body-weight or by the dermal route at a rate of 1.6 ml/kg. Fresh solutions were prepared daily. In tests involving use of the stomach tube, control rats were given plain water. The volume of metepa solution given daily by stomach tube was adjusted each week for changes in body-weight of the rats.

The metepa-poisoned diets were prepared each week. The amount of metepa needed to make the most concentrated diet in use at the time was dissolved in 50 ml of ether and mixed with 50 g of corn starch. The ether was evaporated, and the mixture of metepa and corn starch was added to a sufficient quantity of ground Purina Laboratory Chow [1] to make the desired dietary concentrations. Each of the less concentrated metepa diets was prepared by mixing an appropriate amount of the highest dietary level with sufficient quantity of ground chow. Each batch of diet was thoroughly mixed, using a bakery-type electric mixer, and stored under refrigeration. Ample food was provided for the rats by filling their food cups twice a week. All uneaten food was discarded each time fresh diet was provided.

Blood for white blood cell counts (WBC) and peripheral blood smears was obtained from the rats by tail clipping. The blood smears were stained with Wright's stain. Tissues for microscopic study were fixed in buffered 10% formol, and routine haematoxylin and eosin stains were done.

Acute toxicity

Acute oral and dermal LD_{50} values were determined in adult male rats weighing 256-395 g and in females weighing 185-254 g. The rats were held for a minimum of 22 days for observation following poisoning. The LD_{50} values were calculated by the method of Litchfield & Wilcoxon (1949).

Chronic toxicity

Ninety-six male rats, 4-6 weeks old, were equally distributed into eight groups according to a table of random numbers. Four groups (1-4) were fed metepa in their diet at levels of 0, 200, 400, and 1000 ppm, respectively. The other groups (5-8) were given metapa daily by stomach tube at dosage levels of 0, 5, 10, and 20 mg/kg/day, respectively, for 105 days. White blood counts and differential counts were made on all rats before dosing was begun and on all survivors after 36, 70, and 104 days of dosing. The left testis was removed by surgery for microscopic study from five rats in each group on the 55th day of poisoning, using sodium pentobarbital anaesthesia. The food consumption of all rats in groups 1-4 was measured on days 5-19, 47-53, and 89-100. The surviving rats were sacrificed on the 105th day. They were examined routinely at the time of spontaneous death or sacrifice, and the testes were taken for microscopic study.

To study the spontaneous reversibility of testicular atrophy caused by metepa, 42 male rats, 6-8 weeks old, were randomly distributed into two groups of 30 and 12 each. The first group was fed metepa at a dietary level of 1000 ppm, and the other group (controls) was fed the same diet without added metepa. When poisoning was discontinued on the 66th day, the controls and the rats fed metepa were randomly distributed into three subgroups each to be sacrificed after 0, 50 and 100 days, respectively. At the time of sacrifice the testes were removed, weighed and prepared for microscopic study.

Effect of metepa upon reproduction in male rats

Thirty-five 3.5- to 4.0-month-old male rats of known fertility were distributed into five groups of seven rats each according to a table of random numbers. The five groups of rats (1M to 5M) were given metepa by stomach tube at dosage levels of 0, 0.31, 0.62, 1.25 and 2.50 mg/kg/day, respectively, for 197 days. Beginning on the 15th day of metepa exposure, the males were bred every two weeks through the sixth breeding cycle, and then at less frequent intervals, to non-poisoned females of known fertility. Four groups of 35 females each were used so that no female was bred more often than once in eight weeks. The males and females were mated in pairs and confined together for seven days at each mating. A vaginal smear from each female was examined microscopically early each morning for the presence of sperm for five consecutive days (or less in females inseminated earlier). After mating, the females were caged individually in litter cages. At each successive mating the pairing of the rats was rotated so that no female was ever bred to the

[1] Ralston Purina Co., St. Louis, Mo. Use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service.

TABLE 1
THE ACUTE TOXICITY OF CHEMOSTERILANTS BY THE ORAL AND DERMAL ROUTES IN ADULT RATS

Compound	Route	No.	Sex	LD_{50} (mg/kg)	95 % confidence limits LD_{50} (mg/kg)	Lowest dose (mg/kg)		Survival time (day) [a]	
						Tested	Lethal	Range	Mean
Metepa	Oral	60	M	136	93-199	50	50	3-17	6
		60	F	213	164-277	100	150	2-11	5
	Dermal	50	M	183	156-214	100	150	4-15	7
Tepa	Oral	50	M	37	27- 51	20	20	3-15	7
	Dermal	50	M	87	59-128	50	75	3-17	7
Apholate	Oral	60	M	98	82-118	50	75	3-13	6
		50	F	113	90-141	75	75	3-13	5
	Dermal	70	M	400-800 [b]	—	300	300	2-17	7

[a] Based on rats that died.
[b] Estimated.

same male a second time or to another male in the same group. No female was mated until at least seven days after her last litter was weaned. A sixth group of rats (6M) given 5.00 mg/kg/day was introduced into the experiment after it had been in progress for 112 days. All of the offspring from the males in groups 1M, 4M, 5M, and 6M were autopsied and subjected to a gross examination at the time of sacrifice. At the second mating and at every third breeding thereafter, litters from two males in each of these groups were selected at random and held for observation until they were 90-100 days old. All other litters were destroyed when weaned. At the conclusion of the test, peripheral WBC values were determined on all the survivors in groups 1M-5M, and autopsies were performed.

RESULTS

Acute toxicity

The results of studies on the acute toxicity of metepa, tepa, and apholate are shown in Table 1. The oral toxicity of metepa is about the same as that of DDT but—quite unlike DDT—it is almost as poisonous by the dermal route as when given by stomach tube. Female rats are slightly less susceptible than males to the acute effects of metepa.

Both the oral and dermal toxicities of tepa are similar to those of dieldrin. The oral toxicity of apholate is intermediate between those of metepa and tepa, but it is distinctly less toxic by the dermal route than either of the others.

Time from dosing until death averaged 5-7 days for all three compounds studied and was independent of the route or level of dosage. Typical signs of poisoning were severe diarrhoea, anorexia, and some respiratory difficulty.

Four male and five female rats given approximately an acute LD_{50} dose of metepa (150 mg/kg and 225 mg/kg in males and females, respectively) were sacrificed when their death appeared imminent. The major findings were confined to the gastrointestinal tract, the bone marrow, the spleen, and the lymph-nodes. The epithelium of the gastrointestinal tract, particularly that of the ileum, was degenerated and the wall showed haemosiderin deposits. In addition, fresh haemorrhage was observed in the wall and in the lumen of the ileum and jejunum in some rats. There was pronounced hypoplasia of the bone marrow. The spleen was smaller and weighed less than normal and the lymphocytes appeared to be reduced on microscopic examination. Markedly dilated sinuses were observed in the lymph-nodes, which contained in their stroma many haemosiderin-laden macrophages. Less frequent changes included slight intra-alveolar haemorrhage of the lungs, mild fatty metamorphosis of the liver, and necrosis of the tubular epithelium of the kidney. An effect on the reproductive organs was not observed in the animals killed by an acute toxic dose or in those that survived and were sacrificed within 20 days after such a dose. However, when 45-50-day-old male rats were given a single dose of

100 mg of metepa per kg of body-weight in water by stomach tube and the animals were allowed to live for 50 days before the testes were examined, some of the animals showed either complete or partial testicular atrophy.

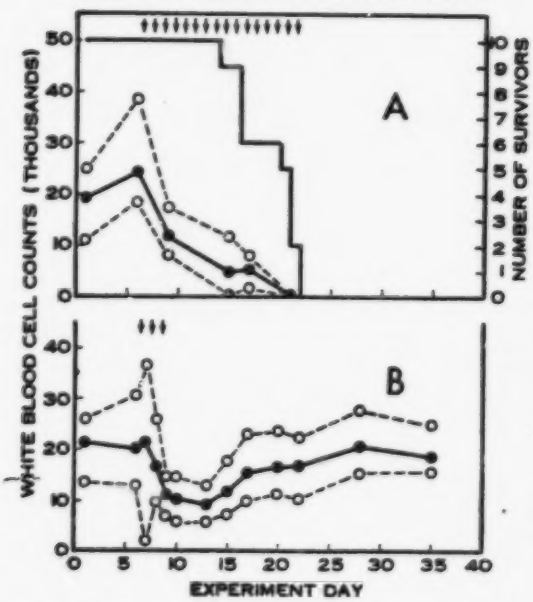
The white blood count as an index of poisoning by metepa

Metepa given by stomach tube at a rate of 50 mg/kg/day (about a third of an LD_{50} dose daily) was rapidly fatal, as shown by Fig. 1A. Eight to 16 doses at this rate were sufficient to kill all of 10 male rats, 90-100 days old. On the contrary, all rats in another group of 10 dosed at the same rate survived when dosage was stopped after the white blood count had fallen precipitously (Fig. 1B). The white count continued to fall for four additional days. However, by the seventh day following the last dose of metepa the white cell count started to recover, and it returned to its pretreatment level by the 20th day. Only three of these rats exhibited any sign of poisoning. All suffered approximately a 15% weight loss from which they recovered by 30 days after the last dose of metepa.

The results of blood studies may serve as a warning of excessive absorption of metepa and thus permit exposure to be stopped before the compound causes serious illness or death.

FIG. 1

WHITE BLOOD CELL COUNTS[a] IN MALE RATS GIVEN METEPA AT A DOSAGE LEVEL OF 50 MG/KG/DAY: (A) UNTIL DEATH OF ALL THE RATS; (B) ONLY UNTIL THE COUNT HAD FALLEN PRECIPITOUSLY (3 DOSES)



[a] High, mean and low values. Each arrow indicates one dose of metepa.

Chronic toxicity

In a preliminary study of the histological effect of metepa, the compound was fed to groups of four male and four female rats at dietary levels of 0, 100, 200, 400, 1000, and 2000 ppm for 108 days. Microscopic study was made of bone marrow smears and of tissue sections of all organs of the genital system, heart, liver, spleen, lungs, kidneys, gastro-intestinal tract, lymph-nodes, and adrenals taken at the time of death of the rats fed 2000 ppm and at the time of sacrifice of the other groups. Brain, salivary glands, and thymus were examined occasionally. The rats fed 1000 and 2000 ppm metepa showed marked hypoplasia of all components of the bone marrow, and this change corresponded to findings in peripheral blood smears. The bone marrow smears from rats fed lower dietary levels of metepa were not different from those of the controls. Except for intra-alveolar haemorrhage into the lungs, particularly in the rats fed the higher dietary levels of metepa, abnormal microscopic findings were confined to the testes and ovaries. The testes were affected in proportion to dietary levels of metepa in rats fed 400 ppm or more, while the ovaries were not affected in rats fed 400 ppm. Consequently, microscopic study of organs other than the testes was not performed in the chronic toxicity studies discussed below.

As shown in Table 2, metepa was more toxic to male rats when given daily by stomach tube in water solution than when given as a component of the diet. The mortality among rats given 10 mg/kg/day, by stomach tube, was greater than among those fed 1000 ppm metepa in the diet (76 mg/kg/day). Part of the difference may be due to the ability of the rat to detoxify a larger dose when it is ingested intermittently during an entire day than when it is given at one time. Rats have survived parathion in their diet for 92 days at a level that gave them a daily dosage equal to 85% of an acute LD_{50} dosage (unpublished data). However, at least a part of the difference for metepa was caused by break-down of the compound in the food before it was ingested by the rats. The colorimetric method of Epstein et al.

TABLE 2

MORTALITY, SURVIVAL TIME AND WEIGHT GAIN IN MALE RATS GIVEN METEPA AS A COMPONENT OF THEIR DIET, OR DAILY IN WATER SOLUTION BY STOMACH TUBE, FOR 105 DAYS

Method of dosing	Group No.	Dosage level (mg/kg/day)	Mortality (died/tested)	Survival time (days) [a]		Body-weight gain (g/rat) of survivors	
				Range	Mean	Range	Mean ± SE
Diet	1	0 [b]	0/12	—	—	230-384	290 ±14
	2	18.2 [b]	0/10	—	—	202-347	271 ±13
	3	32.3 [b]	0/12	—	—	212-342	270 ±15
	4	76.4 [b]	5/12	31-71	56	—9 [c]-167	120 ±22
Stomach tube	5	0	0/12	—	—	203-309	261 ±12
	6	5	1/12	89	89	178-308	251 ±13
	7	10	10/12	44-94	75	163-198	180 ±17
	8	20	12/12	23-46	33	—	—

[a] Based on rats that died.

[b] Groups 1, 2, 3 and 4 were fed metepa at dietary levels of 0, 200, 400, and 1000 ppm, respectively, and dosage level is based on mean food consumption on days 5-19, 47-53 and 89-100.

[c] One animal lost 8 g.

(1954) was used to detect the aziridine ring. All of the metepa was recovered from a sample of the diet analysed on the day of formulation. Only 51% and 35% of the compound could be recovered from the diet after storage under refrigeration for four and seven days, respectively. Only 10% of the metepa could be recovered from the diet following storage at room temperature for four days.

When given by stomach tube, metepa was fatal to most rats at dosages of 10 mg/kg/day or higher, and one of 12 rats died at a dosage of 5 mg/kg/day. Contrary to the effect of a single dose, the time before death occurred was inversely proportional to dosage.

There was a tendency towards a decrease in the weight gain of all the groups of rats given metepa (Table 2). However, only the body-weight gains of the surviving rats in groups 4 and 7 were significantly less than the gains for the controls.

Varying degrees of flaccid paralysis of the hind legs were observed after approximately two months of dosage in the rats fed the 1000 ppm dietary level or in those given 10 mg/kg/day by stomach tube and after about one month in those given 20 mg/kg/day. Microscopic examination of the sciatic nerve of four of these rats revealed myelin degeneration. No other clinical signs of poisoning were noted except for occasional respiratory difficulty and anorexia resulting in rapid weight loss just prior to death of the rats.

Study of the white blood counts and peripheral blood smears of the poisoned rats at intervals indicated that the haematopoietic system was affected only by dosage levels of metepa that killed some rats.

Table 3 (which refers to the same experiment as does Table 2) shows that the lowest dietary level tested was the only dosage level of metepa that failed to cause microscopic testicular atrophy. All other dosage levels caused complete testicular atrophy in some of the rats by 105 days, and some levels caused partial atrophy of the testes in some of the rats by 55 days. All the dosage levels of metepa reduced the weight of the testes; at the higher dosage levels, the weights were approximately a third of normal. Gross examination of the rats at autopsy failed to reveal any damage to other tissues. The atrophic testes showed loss of turgor and a wrinkled outer surface. The testes were brownish tan; no signs of haemorrhage were observed. In all animals showing complete testicular atrophy (see Table 3), the following microscopic changes were observed: the size of the tubules was reduced; their basement membranes were thickened; and the sperms, spermatocytes, and spermatids had disappeared. Spermatogonia remained in some instances but appeared to be reduced. In advanced atrophy only Sertoli cells were seen in the tubules. Interstitial oedema and occasional slight proliferation of interstitial tissue with prominent Leydig's

TABLE 3

MICROSCOPIC TESTICULAR ATROPHY AND WEIGHTS OF TESTES OF RATS GIVEN METEPA DAILY AS A COMPONENT OF THEIR DIET OR BY STOMACH TUBE

Method of dosing	Dosage level (mg/kg/day)	Microscopic testicular atrophy in rats (No. positive/No. examined)				Body and testes weights (g) of rats at death or sacrifice [a]				
		Complete atrophy		Partial atrophy			Body-weight		Testes weight	
		Surgery at 55 days [b]	Death or sacrifice	Surgery at 55 days [b]	Death or sacrifice	No. of rats	Range	Mean ± SE	Range	Mean ± SE
Diet	0 [c]	0/5	0/12	0/5	0/12	7	371-486	444 ± 14	2.35-3.88	3.22 ± 0.21
	16.2 [c]	0/5	0/10	0/5	0/10	5	386-455	431 ± 13	2.04-2.33	2.18 ± 0.22
	32.3 [c]	0/5	1/12	3/5	8/12	7	344-488	411 ± 21	1.30-2.21	1.71 ± 0.12
	76.4 [c]	5/5	11/12	0/5	0/12	7	132-323	225 ± 28	0.71-1.58	0.99 ± 0.12
Stomach tube	0	0/5	0/12	0/5	0/12	6	239-439	384 ± 30	1.92-3.80	3.39 ± 0.19
	5	0/5	2/12	0/5	7/12	7	318-494	398 ± 21	1.15-3.02	1.82 ± 0.28
	10	0/5	9/12	2/5	0/12	7	183-281	213 ± 14	0.56-2.04	1.21 ± 0.23
	20	—	5/12	—	3/12	12	96-141	121 ± 5	0.46-1.90	1.19 ± 0.13

[a] Does not include rats that had one testis removed after 55 days' exposure.

[b] Based on examination of one testis removed by surgery.

[c] Groups 1, 2, 3 and 4 were fed metepa at dietary levels of 0, 200, 400, and 1000 ppm, respectively; dosage level is based on mean food consumption on days 5-19, 47-53 and 89-100.

cells were observed. Sometimes the lumen or the periphery of the tubules contained multinucleated giant cells with either very small pyknotic nuclei or enlarged nuclei of empty appearance and highly acidophilic cytoplasm. At the lower dosage levels—and after a shorter period of exposure even at the higher dosage levels—these tissue alterations were less severe (Table 3). Testes that showed only partial atrophy still contained several tubules with normal-appearing spermatogenesis. If the testes were damaged to an even lesser degree, only the number of cells within the tubules appeared to be reduced. However, this reduction is difficult to evaluate since it is subject to many artefacts, particularly variations in the thickness of the section. Therefore, it is worth while to examine several sections before arriving at the conclusion that there is a reduction in spermatogenesis. The 12 controls that had received plain feed and the 12 controls that were given plain water daily by the stomach tube had completely normal testes at gross and microscopic examination. The microscopic changes seen in the atrophic testes were not specific for rats dosed with metepa, but have been described following cadmium, inanition, vitamin E deficiency, and other environmental factors (Ribelin, 1963).

The degree of spontaneous reversibility of complete microscopic testicular atrophy produced in rats by high dietary levels of metepa may be demonstrated by the decrease in the percentage of rats affected as the length of time between exposure and examination is increased (Table 4). The dosage level of metepa, based on measurement of food consumption the first two weeks of poisoning, was 89 mg/kg/day. Under some conditions of poisoning, some rats are able to recover from complete testicular atrophy caused by metepa, but recovery occurs slowly at a dosage level (1000 ppm) known to be large enough to kill some rats.

Effect of metepa upon reproduction in male rats

Since a dosage level of 5.00 mg/kg/day given by stomach tube caused testicular atrophy in some rats dosed for 105 days, it was thought that even smaller dosage levels might produce a functional effect upon the genital system that would not be detected by histological study. Consequently, groups of seven male rats were given metepa by stomach tube at dosage levels of 0, 0.31, 0.62, 1.25 and 2.50 mg/kg/day, respectively, and bred at intervals to non-poisoned females. Beginning with the second week of poisoning, the rats were bred a total of 10 times

TABLE 4

THE SPONTANEOUS REVERSIBILITY OF TESTICULAR ATROPHY IN RATS FED METEPA AT A DIETARY LEVEL OF 1000 ppm FOR 66 DAYS

Dietary level (ppm)	Days from exposure to examination	Testicular atrophy		Body-weight (g)		Testes weight (g)		Testes weight as % body-weight
		Rats examined	Rats affected (%)	Range	Mean ± SE	Range	Mean ± SE	
1 000	0	10	100	264-320	288 ± 7.0	0.84-1.40	1.12 ±0.05	0.39
Control	—	4	0	325-417	366 ±18.0	3.06-3.82	3.35 ±0.12	0.92
1 000	50	9	56	348-435	378 ± 9.3	1.16-2.49	1.48 ±0.13	0.39
Control	—	4	0	380-518	421 ±33.9	2.93-3.53	3.31 ±0.19	0.79
1 000	100	9	44	388-545	454 ±18.1	1.25-2.98	1.97 ±0.20	0.43
Control	—	4	0	403-485	454 ±19.2	2.96-5.10	3.84 ±0.46	0.86

over a period of 197 days. Rats given 5.00 mg/kg/day were introduced into the experiment after it had been in progress for 112 days. They were compared with a separate group of controls. However, since there was no appreciable difference in the litter production in the two control groups, only the control group of males set up at the start of the study is included in Tables 5 and 6 and in Fig. 2.

Results presented in Table 5 indicate that the proportion of successful matings was severely reduced for rats given metepa at a rate of 5.00 mg/kg/day. In fact, no litters resulted from the breeding of these males after 70 doses, although six of the seven females bred were inseminated and one vaginal smear contained motile sperm. Even when litters were born, the number of offspring per litter was greatly reduced by a dosage of 5.00 mg/kg/day and somewhat reduced by a dosage of 2.50 mg/kg/day ($P < 0.001$). It is apparent from Fig. 2 that this reduction in offspring per litter occurred in less than 22 days at a dosage of 5.00 mg/kg/day and in about 80 days at a dosage of 2.50 mg/kg/day. The survival of young was apparently not affected even at a dosage of 5.00 mg/kg/day.

Values for white blood cell counts and testis weights of the poisoned rats at the time of sacrifice are shown in Table 6. There was no correlation between the metepa dosage level and the white

TABLE 5

SUMMARY OF REPRODUCTION AND SURVIVAL OF OFFSPRING FROM MALE RATS GIVEN METEPA DAILY BY STOMACH TUBE FOR 197 DAYS [a]

Metepa dosage level (mg/kg/day)	No. of test male survivors [b]	Total No. of matings	Total No. of litters born alive	Ratio of No. of litters: No. of matings (%)	Average No. per litter		Survival (%)
					Born alive	Weaned	
0	7	70	62	89	10.7	10.0	94
0.31	5	62	54	87	9.9	9.1	92
0.62	6	62	58	94	9.9	9.4	95
1.25	6	62	47	76	9.5	8.8	93
2.50	5	38	47	81	7.7	7.3	95
5.00 [c]	7	21	5	24	3.2	2.8	88

[a] Values based on results for 10 mating periods during metepa exposure.
[b] Started with 7 rats in each group but some died of stomach tube injury during course of study.
[c] Dosed for only 77 days and mated after 22, 41 and 70 doses only.

TABLE 6

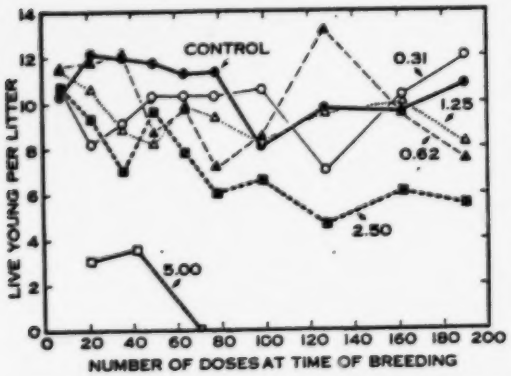
FINAL WHITE BLOOD COUNT (WBC), BODY-WEIGHT, AND TESTICULAR WEIGHT OF RATS GIVEN METEPA DAILY BY STOMACH TUBE FOR 197 DAYS

Metepa dosage level (mg/kg/day)	Final WBC (× 1 000)		Final body-weights (g)		Testes weight (g)		Testes weight as % of body-weight
	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	
0	8.0-25.4	15.3 ± 2.0	433-604	517 ± 22.0	3.44-4.19	3.84 ± 0.09	0.70
0.31	12.3-22.5	16.6 ± 1.8	486-694	592 ± 43.0	3.21-3.92	3.69 ± 0.13	0.62
0.62	8.1-25.2	17.1 ± 2.3	319-514	446 ± 27.7	3.06-3.80	3.50 ± 0.12	0.78
1.25	11.7-38.3	22.5 ± 3.4	402-584	488 ± 27.5	2.94-4.02	3.29 ± 0.17	0.67
2.50	9.8-17.5	13.6 ± 1.3	488-546	508 ± 17.6	2.20-2.46	2.37 ± 0.04	0.47
5.00[a]	—	—	323-450	412 ± 17.1	1.24-2.08	1.69 ± 0.10	0.41

[a] Dosed for only 77 days.

FIG. 2

NUMBER OF OFFSPRING BORN ALIVE PER LITTER TO FEMALE RATS BRED TO MALE RATS POISONED BY METEPA[a]



[a] Each line corresponds to the metepa dosage level indicated.

count. The blood differential values were within normal limits. The testis weights, as a percentage of body-weight, of the metepa-poisoned rats given 2.50 and 5.00 mg/kg/day were significantly smaller than those of the controls ($P < 0.001$).

Microscopic examination of sections of lymph-nodes, thymus, spleen, heart, kidneys, trachea, lungs, gastrointestinal tract, salivary glands, liver, adrenals, bladder, epididymis, seminal vesicles, and testes showed that the only organ definitely affected was the testis. One of five rats given 2.5 mg/kg/day of metepa suffered partial atrophy of the testes in 197 days, and all seven given 5.00 mg/kg/day for 77 days showed partial to complete atrophy. No testicular atrophy was detected in any of the rats given less than 2.50 mg/kg/day.

All weanlings were examined clinically, and autopsies were done on all from groups 1M, 4M, 5M, and 6M. Two instances of hydrocephalus were found. This phenomenon will require further examination since it occurs spontaneously in our colony. Following the third, sixth, and ninth matings of the poisoned males, after they had been dosed for 36, 78, and 162 days, respectively, the litters from two males of each group given 1.25 and 2.50 mg/kg/day of metepa and from two control males were saved for observation until they were 90-100 days old. These litters developed normally, and there was no evidence of any abnormalities in any of the organs at autopsy. Out of a total of 2588 offspring, 142 died spontaneously; they were autopsied and the organs subjected to gross examination, but no significant changes were seen.

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RÉSUMÉ

Certains agents alcoylants, qui possèdent des propriétés stérilisantes, sont susceptibles d'être utilisés dans la lutte biologique contre les insectes vecteurs. Le présent travail rapporte les observations faites chez le rat au cours d'expériences portant sur la toxicité et l'action stérilisante de trois de ces composés, le metepa, le tepa et l'apholate, principalement du metepa.

La toxicité du metepa et de l'apholate administrés en une dose unique par voie orale est très semblable à celle du DDT; la toxicité du tepa est voisine de celle de la dieldrine. Le metepa et le tepa sont également très toxiques s'ils sont introduits dans l'organisme par voie dermique.

L'administration répétée de petites doses de metepa lèse en premier lieu le testicule; les ovaires et la moelle osseuse ne sont atteints que si l'on utilise des dosages plus élevés et il faut des doses importantes pour provoquer des lésions de l'épithélium intestinal et des tissus lymphoïdes. Quant aux éléments blancs du sang, leur nombre ne diminue que si l'on administre de façon répétée des doses finalement létales. L'arrêt de la médication au moment où s'amorce la chute brutale du nombre des leucocytes permet d'assister à un lent retour à la normale. La morphologie des cellules et la formule leucocytaire ne sont pas modifiées.

Les lésions testiculaires ont été spécialement étudiées. Donné à la dose de 5 mg/kg/j, soit environ 4% de la DL_{50} par voie orale, le metepa provoque l'atrophie testiculaire chez le rat mâle en 77 jours, et en 55 jours si la dose est portée à 10 mg. Chez certains animaux, l'administration quotidienne de 2,5 mg du produit pendant 197 jours entraîne une atrophie partielle des testicules et une dose journalière de 1,25 mg/kg reste sans effet. Si les doses ne sont pas trop élevées et, vraisemblablement, si l'on cesse de donner le metepa après un certain temps, on peut assister à une régénération de l'épithélium tubulaire des testicules.

Pour une dose quotidienne de 5 mg/kg incorporée à la nourriture des rats mâles, l'effet sur la capacité de reproduction de l'espèce se manifeste par une réduction sensible du nombre et de l'importance des portées. Cette limitation apparaît dès le 22e jour et aboutit finalement à la stérilité complète. Les mêmes résultats, moins accentués cependant, s'observent si la dose est réduite à 2,5 mg: le produit doit être administré dans ce cas pendant 80 jours, et pendant les 197 jours d'expérimentation, on n'a pas constaté de stérilité complète. Dans aucun cas, les possibilités de survie de la progéniture n'ont été affectées.

Chez les rats mâles d'un autre groupe recevant quotidiennement 5 mg/kg de metepa, pendant 55 jours, on n'a pas observé d'atrophie testiculaire. Il semble donc que la stérilité soit due, au début, à des troubles fonctionnels qui précèdent l'installation des lésions décelables à l'examen microscopique courant.

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Toxicity of Fentin Hydroxide to Rats

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Kenaga (1965) reported that some triphenyl tin compounds sterilize house flies, German cockroaches, and confused flour beetles. The most active compound among these reproduction inhibitors was fentin hydroxide (triphenyl tin hydroxide).

Little is known about the toxicity and the metabolism of triphenyl tin compounds. The fungicidal effect of triaryl tin compounds was shown by Härtel (1958), and a preparation of triphenyl tin was registered by Farbwerke Hoechst, Germany, under the trade name Brestan. Barnes and his group (Barnes and Stoner, 1958, 1959; Magee *et al.*, 1957; Stoner, 1966) and Verschuuren *et al.* (1966) have studied the toxicity of various tin derivatives, mainly alkyl tin compounds. Klimmer (1964) reported on the toxicity of fentin acetate (triphenyl tin acetate) in rats, guinea pigs, and rabbits.

The purpose of this paper is to describe the acute toxicity of fentin hydroxide in male and female rats and its subacute toxicity and effect on fertility in male rats.

METHODS

Sherman strain rats were used, and essentially the same methods were employed as described previously for other studies of reproduction (Gaines and Kimbrough, 1964). With specified exceptions, the rats were individually caged and were weighed at weekly intervals. The fentin hydroxide used was technical grade[1] and was suspended in peanut oil when given by stomach tube and the control rats were given only peanut oil by the same route. The diets were prepared by grinding a predetermined amount of fentin hydroxide with 10 g of corn starch using a mortar and pestle. This material was mixed with 415 g of ground laboratory chow and then mixed with the proper quantity of ground chow to give the most concentrated diet used. Diets containing lower concentrations of fentin hydroxide were made by diluting further a portion of the next higher concentration with ground chow. All diets were mixed with a mechanical mixer.

Blood for leukocyte counts (WBC) and peripheral blood smears was obtained from the rats by tail clipping. Tissues for microscopic study were fixed in buffered 10% formalin, except for the brain, testes, and epididymis, which were fixed in Bouin's solution. Routine hematoxylin and eosin stains were performed.

The acute oral toxicity was determined in 40 female and 70 male rats. The rats were 4–5 months old; the males weighed 286–522 g and the females 196–312 g. They were

[1] The fentin hydroxide was kindly supplied by Dr. E. E. Kenaga, Dow Chemical Co., Midland, Michigan.

held for observation for a minimum of 15 days after poisoning. The LD_{50} values were calculated by the method of Litchfield and Wilcoxon (1949).

Forty 4- to 5-week-old male rats were divided into 4 groups of 10 animals each, according to a table of random numbers. The animals were fed the compound in the diet at levels of 0, 100, 200, and 400 ppm. Food consumption was measured during weeks 1, 2, 7, 8, 13, and 14 of exposure. All surviving animals were sacrificed 99 days after onset of exposure, and complete autopsies were performed. Prior to sacrifice, a WBC and differential were performed on blood from these rats.

For study of effects on fertility of males forty 8- to 9-week-old rats were distributed into 4 groups of 10 rats each, according to a table of random numbers. They were given fentin hydroxide in the diet at levels of 0, 50, 100, and 200 ppm. During the mating period, the animals were given the compound by stomach tube so the females received no poison and both males and females had constant access to food. The dosage given by stomach tube was based on the mean fentin hydroxide consumption (mg/kg/day) for the previous week. The food consumption was measured during the first and fourth week of the experiment and thereafter always during the week prior to mating. The rats were mated for the first time on day 29 of exposure to the chemical and then again on days 64, 113, 170, and 238. The males and females were pair-mated and left together for 7 days. During the mating period, early morning vaginal smears were checked for the presence of sperm. The offspring were kept until weaning and checked grossly for abnormalities.

Leukocyte count, differential, and a hemoglobin determination were performed on each animal 269 days after onset of exposure to fentin hydroxide. All surviving male rats were sacrificed 276 days after onset of the experiment, and complete autopsies were performed.

RESULTS

The oral LD_{50} value for fentin hydroxide in adult rats was 240 mg/kg, with 19/20 confidence limits of 189–305 mg/kg, for males and 360 mg/kg, with 19/20 confidence limits of 283–457 mg/kg, for females.

The time interval between dosing and death ranged from 1–16 days in both sexes. Symptoms of poisoning were sluggishness, unsteadiness, moderate diarrhea, anorexia, bloody stain around the nose and eyes, and wheezing.

Autopsies were performed on 8 male rats that died and 31 males and 7 females that were sacrificed 17–27 days after a single dose of fentin hydroxide given by stomach tube. The doses in the males ranged from 150–400 mg/kg, while 6 females received 300 mg/kg and 1 female, 600 mg/kg. The organs of the rats that survived were normal grossly, and microscopic examination in these was limited to the reproductive organs, heart, and muscle. Tissue of brain, lungs, salivary gland, spleen, kidney, liver, adrenal, and heart was examined microscopically in the 8 rats that died. All of the examined organs of the females were normal grossly and microscopically. Extensive intra-alveolar hemorrhage and pulmonary edema were observed in the male rats that died. Two of these had an inflammatory process in the lungs. The testes of 3 of the males that died and 26 of the survivors had some atypical spermatids, never very pronounced. No other changes were observed grossly or microscopically in the rats.

The weanling male rats in the subacute toxicity study received fentin hydroxide in

TABLE 1

MEAN FOOD CONSUMPTION (G/KG/DAY) AND MEAN BODY WEIGHT GAIN (G) AT VARIOUS INTERVALS IN THE SUBACUTE TOXICITY AND REPRODUCTION STUDIES IN RATS FED FENTIN HYDROXIDE

Fentin hydroxide (ppm)	Parameter	Subacute						Reproduction					
		Week 1	Week 2	Week 7	Week 8	Week 13	Week 14	Week 1	Week 4	Week 9	Week 16	Week 24	Week 34[a]
0	Food	131.8	102.7	68.2	66.8	61.2	59.2	88.8	70.6	61.3	53.9	48.3	50.2
	Wt. gain	21	43	145	19	62	12	11	77	75	57	46	23
50	Food	—	—	—	—	—	—	87.7	68.5	61.6	52.7	48.1	49.6
	Wt. gain	—	—	—	—	—	—	16	77	77	39	34	17
100	Food	97.7	101.7	71.4	70.4	55.4	53.5	72.2	72.8	61.0	55.8	47.3	47.7
	Wt. gain	11	42	168	22	68	13	2	73	67	42	23	11
200	Food	66.4	96.5	74.6	77.4	61.5	62.7	46.2	70.8	62.2	58.4	50.0	53.4
	Wt. gain	−4	24	113	19	68	11	−14	42	59	31	22	20
400	Food	40.9	82.1	—	—	—	—	—	—	—	—	—	—
	Wt. gain	−16	−12	—	—	—	—	—	—	—	—	—	—

[a] Last food measurement although rats were fed diet for 276 days.

TABLE 2

SUMMARY OF REPRODUCTION AND SURVIVAL OF OFFSPRING FROM MALE RATS FED FENTIN HYDROXIDE IN THEIR DIET

Dietary level (ppm)	Number of test male survivors	Total number of matings	Total number of litters born alive	Ratio of number of litters to number of matings (%)	Average number per litter		Survival (%)
					Born alive	Weaned	
0	9	48	37	77	11.7	10.9	93
50	10	50	43	86	12.0	10.9	91
100	10	50	36	72	11.0	10.5	95
200	7	42	27	64	10.1	9.1	90

the diet at levels of 0, 100, 200, and 400 ppm for 99 days (see Table 1). The rats fed 400 ppm were apparently repelled by the compound since they ate only about one-third as much food (41 g/kg/day) as the controls (132 g/kg/day) during the first week. All the animals in this group died in 7–34 days. As shown in Table 1, the rats fed 200 ppm gained less weight (231 g) than the controls (302 g). At the end of the study, the total WBC (11,050) was significantly below that of the controls (15,095). The rats fed 100 ppm appeared normal throughout the study.

Autopsies performed on 6 of 10 rats fed 400 ppm of fentin hydroxide showed extensive intra-alveolar hemorrhage in the lungs of 5 rats. All 6 rats had either partial or complete testicular atrophy. Generally the organs of these animals were smaller than those of the controls. The spleens and the kidneys of the rats fed 200 ppm of fentin hydroxide also weighed less than those of the controls ($p < 0.025$). No other abnormalities were seen grossly or microscopically in the rats fed 200 and 100 ppm of fentin hydroxide.

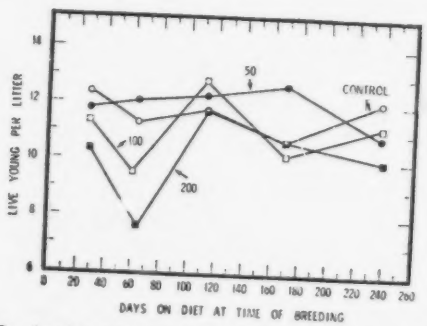

FIG. 1. Number of offspring born alive per litter to female rats bred to male rats poisoned by fentin hydroxide. Each line corresponds to the fentin hydroxide dietary level indicated.

Since all of the male rats fed 400 ppm of fentin hydroxide in the study on subacute toxicity showed partial or complete testicular atrophy 7–34 days after onset of the experiment, it was thought that even smaller dosage levels might produce a functional effect upon the genital system. Thus, 4 groups of 10 rats each were fed 0, 50, 100, and 200 ppm of fentin hydroxide in the diet and were mated at intervals to nonpoisoned females. Results shown in Table 2 and Fig. 1 indicate that, in comparison to the controls, there was a drop of about 27 and 18%, respectively, in the total number of offspring in the groups fed 200 and 100 ppm after 64 days of exposure. However, when the males were subsequently mated, their fertility improved. Leukocyte counts, differentials, and hemoglobin determinations performed 1 week prior to the time of sacrifice did not differ from the controls. Gross abnormalities related to fentin hydroxide were not observed in the offspring.

In the group fed 200 ppm, 1 rat died and 2 were sacrificed when moribund. The cause of death in this group was generalized internal hemorrhage. In the animals that were sacrificed, no gross or microscopic abnormalities attributable to fentin hydroxide were observed.

In the reproduction study, as in the study on subacute toxicity, the food consumption of the animals receiving a diet containing fentin hydroxide, particularly at higher

dosage levels, was significantly lower during the first week of the experiment, and then it improved for all dosage levels (see Table 1).

DISCUSSION

Fentin acetate and triphenyl tin chloride are not very palatable to rats, as pointed out by Ascher and Nissim (1964), who recommended these compounds as rodent repellents. In studies on the toxicity of fentin hydroxide in another laboratory, dosages of 50 ppm had an effect on the food intake and growth rate of male rats (Verschuuren *et al.*, 1966). Our study indicates that 100 ppm or more of fentin hydroxide in the diet reduces the food consumption of rats, and thus plays a part in the toxic effect of this compound. In the reproduction study, reduced fertility was observed after 64 days of exposure in those rats given either 200 or 100 ppm of fentin hydroxide in their diet (Table 2). It was during this early period, however, that the food consumption of these animals was markedly reduced (see Table 1). The food consumption later improved and with it the fertility, so that at the end of the experiment, the fertility of the animals fed fentin hydroxide did not differ from that of the controls. It has been pointed out repeatedly that starvation affects the testes in rats (Ribelin, 1963), causing a reduction in spermatocytes, spermatids, and spermatozoa. Thus, the partial starvation of the animals in the early part of the experiment probably caused the reduced fertility and probably accounted for the testicular atrophy observed in the rats fed 400 ppm in the study on subacute toxicity.

Klimmer (1964) found the acute and chronic oral toxicity of fentin acetate to be somewhat greater than what we obtained for fentin hydroxide.

The absorption of triphenyl tin compounds from the gastrointestinal tract is poor. After ingestion of triphenyl tin by cows and sheep, the compound is largely excreted with the feces (Brüggemann *et al.*, 1964; Herok and Götte, 1964). Probably because of greater absorption, the acute intraperitoneal toxicity of triphenyl tin compounds is much greater than the acute oral toxicity (Stoner, 1966).

Light microscopic studies of the various organs of exposed animals did not reveal any abnormalities that could be related to fentin hydroxide. Morphologic changes recorded for other organic tin compounds include bile duct lesions produced by diethyl tin salts (Barnes and Stoner, 1958) and interstitial edema of the white matter of the brain and spinal cord produced by trialkyl tin compounds (Magee *et al.*, 1957). Atrophy of the testes and accessory genital organs was observed by Magee *et al.* (1957) in some rats which had received 10–20 ppm of triethyl tin hydroxide for 3–5 months. None of these lesions was observed in our feeding experiments with fentin hydroxide, except that testicular atrophy (apparently caused by starvation) was present at a dietary level of 400 ppm. Klimmer (1964) studied the acute and chronic oral toxicity of fentin acetate in rats and also emphasized the lack of pathologic changes in these animals.

SUMMARY

Fentin hydroxide (triphenyl tin hydroxide) was given in the diet to rats at the rate of 400 ppm. This amount reduced the food consumption and, within 7–34 days, caused death from starvation and hemorrhage in all tested rats. Dietary levels of 100 and 200 ppm reduced food consumption and weight gain, particularly during the first week. By week 7, the animals had

adapted to these concentrations of the chemical, and the food consumption was the same as that of the controls. The food intake or weight gain was not affected in rats fed 50 ppm of fentin hydroxide for 276 days. After 64 days of dietary exposure at 200 or 100 ppm, reduced fertility was observed in male rats, but fertility improved later, and was comparable to that of the control rats by day 113. The difference was explained by the partial starvation which the animals suffered, particularly during the first week of the experiment. Gross and microscopic examination of rats fed fentin hydroxide at the rate of 200, 100, or 50 ppm for 276 days did not reveal changes in any organ that could be related to the compound.

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The Sterilizing, Carcinogenic and Teratogenic Effects of Metepa in Rats

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Certain alkylating agents show promise as chemicals for the sterilization of insects. Of these, metepa (tris[1-2(2-methylaziridinyl)] phosphine oxide) is the compound that has received the most study.[a] The acute and subacute toxicity of this compound in Sherman-strain rats has been reported previously.[b] This earlier study indicated a cumulative toxic effect of the compound or its metabolites on the rat testis. Therefore, the chronic toxicity of this alkylating agent was investigated further.

[a] Smith, C. N. (1963) *Bull. Wld Hlth Org.*, **29**, Suppl., p. 99.

[b] Gaines, T. B. & Kimbrough, R. D. (1964) *Bull. Wld Hlth Org.*, **31**, 737.

Materials and methods

A total of 95 male Sherman-strain rats 5-6 weeks old at the beginning of the experiment were caged individually and given a commercial laboratory-animal ration (Purina Laboratory Chow) and water *ad libitum*. The rats were divided into four groups of 20 and one group of 15. Three groups of 20 animals were given a water solution of metepa by stomach tube at dosages of 0.625 mg/kg/day, 2.5 mg/kg/day and 5.0 mg/kg/day. There were two control groups; 20 rats were dosed daily by stomach tube with tap-water, and the remaining 15 animals received no treatment whatever but served as a check on the process of stomach-tubing. At the age of 90-100 days, 12 rats from each of the five groups were chosen at random and mated individually to young adult females of previously established fertility. The remaining rats in each group were held in reserve to replace any of the animals in the breeding experiment that might die. The animals that received metepa at the rate of 0.625 mg/kg/day or 2.5 mg/kg/day were given the compound for eight weeks before they were mated for the first time, and they continued to receive the compound until the end of the experiment; that is, after 422 doses given over the same number of days. The animals given 5 mg/kg/day were dosed for only three weeks prior to the first mating. This group was dosed for a total of only 155 days and then observed for possible recovery of fertility.

The methods employed for breeding and examining rats have been described previously in greater detail.[b] Briefly, the animals were mated at intervals of four to eight weeks. They were pair-caged for 10 days, and early-morning vaginal smears were examined for the presence of sperm. The offspring were examined clinically after birth and at weaning.

Total white-blood-cell counts were performed just prior to the first mating and about six and 10 months after the onset of the experiment. All adult male rats that died during the experiment were autopsied, the tissues were fixed in neutral 10% formol, and sections were stained with routine hematoxylin-eosin stain.

The possibility that metepa has teratogenic properties was investigated also. For this purpose, female rats were given either a single intraperitoneal injection of metepa dissolved in normal saline on the twelfth day of pregnancy or daily intraperitoneal injections on the seventh through thirteenth days of pregnancy. An equal number of pregnant control rats were injected intraperitoneally with normal saline according to the same schedule. The first day of pregnancy was established by examining vaginal smears for sperm. On the twentieth day of pregnancy, counting the first day of pregnancy as day zero, the rats were anaesthetized with ether and the offspring removed by caesarean section. The methods outlined by Wilson[c] were followed in examining the young. Briefly, all foetuses and placentas were blotted dry and weighed immediately after their removal from the uterus. All the young were examined, and then about half of them were fixed in Bouin's solution and later serially sectioned by hand and examined under the dissecting microscope. The remaining animals were fixed in 95% ethyl alcohol and examined for anomalies of the skeleton after clearing the tissue with potassium hydroxide and staining the bones with alizarin red.

Results and comment

In the tests in which male rats were given metepa by stomach tube, the total number of litters and the average number of offspring per litter born alive were reduced in the groups dosed at the rates of 2.5 mg/kg/day and 5.0 mg/kg/day, but the survival rate of the offspring was not affected (see the table). The number of animals born alive was significantly reduced even in the first offspring of the males that received these dosages when they were compared with the animals given tap-water by stomach tube (73 offspring from the 2.5-mg/kg/day group in the first litters and 64 from the 5.0-mg/kg/day group, compared with 113 from sires given tap-water injections; $P = < 0.01$ and < 0.001). As shown in the accompanying figure, there was a small and gradual reduction of the fertility of the controls (attributable largely to the females). However, in addition to this small change in the control group and in addition to the reduction of fertility observed at the first mating, there was a progressive reduction of fertility at subsequent matings in rats dosed at the rates of 2.5 mg/kg/day and 5.0 mg/kg/day. The progressive decrease was more marked in connexion with the number of litters produced than with the number of live young in the litters that were born. There were occasional stillbirths, but the proportion of these from females mated with males treated with metepa was no greater than that from females mated with the control males. Within six months after dosing

[c] Wilson, J. G. (1965) *Methods for administering agents and detecting malformations in experimental animals.* In: Wilson, J. G. & Warkany, J., ed., *Teratology, principles and techniques*, Chicago, University of Chicago Press, pp. 262-277.

SUMMARY OF REPRODUCTION AND SURVIVAL FROM MALE RATS GIVEN METEPA DAILY BY STOMACH TUBE FOR 315 DAYS [a]

Metepa dosage level (mg/kg/day)	No. of test males	Total No. of matings	Total No. of litters born alive	Ratio: No. of litters / No. of matings (%)	Average No. per litter		Survival (%)
					Born alive	Weaned	
0	12	72	56	78	10.8	9.6	89
0	12	72	51	71	9.8	8.9	91
0.625	12	72	56	78	9.5	9.1	96
2.5	12	72	45	63	5.8	5.0	89
5.0 [b]	12	80	26	43	4.8	4.5	94

[a] Values based on results for seven mating periods during metepa exposure.

[b] The group receiving 5 mg/kg/day of metepa was exposed for 140 days at the time of the fifth mating, which produced only one litter from 12 matings. Metepa was then discontinued after 155 days. Matings at 4 and 11 weeks showed little or no improvement in fertility, but complete recovery of fertility occurred within 6 months.

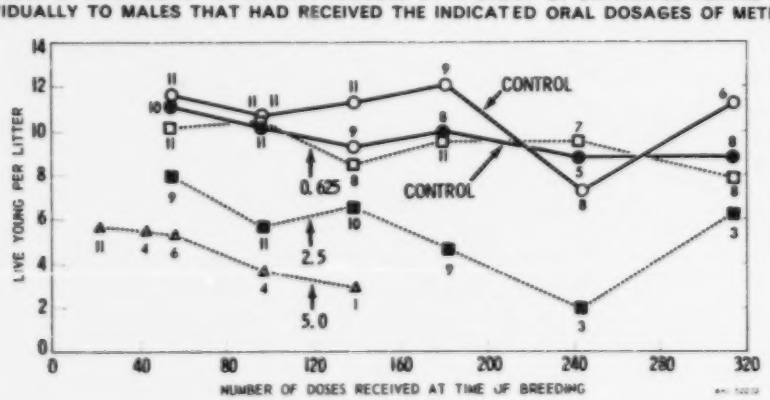
was stopped in the group receiving 5.0 mg/kg/day, the fertility of these animals had returned to normal.

The animals given 0.625 mg/kg/day did not show any diminution of fertility as compared with controls even after a total exposure of 45 weeks. The results regarding fertility in this experiment are consistent with those reported earlier in the shorter-term experiment.[b]

The mean total white-blood-cell counts were lower in the rats given metepa at the rate of 5 mg/kg/day at the end of their dosage period and in those given 2.5 mg/kg/day after about 10 months than the counts in the control rats; mean values were 13 280, 14 910 and 18 780, respectively. However, the differences were not statistically significant.

Five of the 20 rats given tap-water daily by stomach tube and one of 20 rats given 0.625 mg/kg/day of metepa died during the course of the study. One of the rats given water only and the one given metepa both died of stomach-tube injury. Eleven of 20 rats given metepa at the rate of 2.5 mg/kg/day died, as did 12 of 20 rats given 5 mg/kg/day. The deaths of five rats in the former group and two in the latter group were caused by stomach-tube injury. Three lymphatic leukaemias occurred in the group that was given metepa at the rate of 2.5 mg/kg/day.

AVERAGE NUMBER OF LIVE YOUNG PER LITTER PRODUCED BY A GROUP OF UNTREATED FEMALE RATS MATED INDIVIDUALLY TO MALES THAT HAD RECEIVED THE INDICATED ORAL DOSAGES OF METEPA [a]



[a] The figure at each point indicates the number of litters produced from 12 matings.

Two other lymphatic leukaemias, one chloroleukaemia, and a brain tumour occurred in the animals given 5 mg/kg/day. The first animal to develop leukaemia in this latter group was seven months old at the time of death. The other of the two last-mentioned lymphatic leukaemias was successfully transplanted into six of eight three-day-old suckling rats by injecting them with 0.2 ml of homogenized spleen intraperitoneally.

The brain tumour (an astrocytoma) occurred in a male rat that was about 12 months old at the time of death. Grossly, the soft, tan-coloured tumour, which measured about 0.4 × 0.5 × 0.7 cm, occupied the anterior third of both cerebral hemispheres. Microscopically, it consisted of fairly uniform cells with round nuclei, a few of which contained mitotic figures. The cells were separated from each other by a moderate amount of extracellular glial fibrils.

Spontaneously occurring leukaemias and brain tumours are rare in rats [d-f] and have not been observed to occur spontaneously in our colony. However, brain tumours have occasionally been induced in rats by systemically administered alkylating agents such as methylnitrosourea and 2,7-bis-acetylaminofluorene. [g, h] The ability of alkylating agents to produce a variety of other tumours, particularly at the site of application, was reviewed recently.[i]

In the tests involving intraperitoneal injection of female rats, metepa was shown to be teratogenic. Following injection at the rate of 30 mg/kg on the twelfth day of pregnancy, six female rats produced 40 resorptions and 43 foetuses, 16 of which were dead. All of the 27 live and 16 dead foetuses had malformations, some multiple. The incidence of ectrodactylia was 100% in the front paws and 33% in the hind paws. One foetus had hydrocephalus. Two instances of webbed toes in the hind paws, four instances of meningocele, and five instances of kinky tail were encountered. The average birth weight of the foetuses (1.47 g) was significantly reduced (3.44 g as against 1.47 g; $P < 0.001$) and the average weight of the placentas was also significantly lower (0.53 g as against 0.20 g; $P < 0.001$). When daily injections of metepa were given intraperitoneally on days 7 through 13 of pregnancy, the injury detected at the lowest dosage was reduction of the average weights of the foetuses and the placentas. Even a dosage of 1.25 mg/kg daily for seven days resulted in a significant reduction in the weight of the foetus (3.50 g as against 3.22 g; $P < 0.001$) and the placenta (0.558 g as against 0.451 g; $P < 0.001$).

In summary, the sterilizing effect of metepa in male rats was confirmed, and carcinogenic and teratogenic properties of the compound were established. Whether the teratogenic potential is the one to which the rat is the most sensitive cannot be determined from this study, since the route of administration of the compound varied.

[d] Cohrs, P., Jaffé, R. & Meessen, H. (1958) *Pathologie der Laboratoriumstiere*, Berlin, Springer Verlag, vol. 1, p. 782.

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[f] Kimbrough, R., Gaines, T. B. & Sherman, J. D. (1964) *J. nat. Cancer Inst.*, **33**, 215.

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[h] Snell, K. G., Stewart, H. L., Morris, H. P., Wagner, B. P. & Ray, F. E. (1961) *J. nat. Cancer Inst.*, **5**, 85.

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PUBLICATION

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Poisoning by Organic Phosphorus Pesticides Potentiated by Phenothiazine Derivatives

Abstract. Repeated administration of the phenothiazine derivatives chlorpromazine and promazine increased the toxicity of a single dose of parathion in male rats. In female rats, a single dose of 5 mg/kg of promazine increased the toxicity of a single dose of parathion, but had no apparent effect on the toxicity of a single dose of Phosdrin. Repeated dosage with chlorpromazine and atropine, or promazine and atropine, after administration of parathion at the same rate, resulted in a slightly higher mortality in female rats than did atropine or no treatment.

In human patients and small animals phenothiazine tranquilizers (*1*) often increase the effectiveness of sedatives, analgesics, and narcotics (*2*). The possibility that promazine may potentiate poisoning in man by organic phosphorus pesticides was suggested by Arterberry *et al.* (*3*). On the other hand, Frada and Gucciardi (*4*) reported that treatment with chlorpromazine prolonged the survival time of guinea pigs poisoned with a mixture of malathion and parathion, but they did not indicate whether any of their treated animals survived. Other investigators have reported that a combination of atropine and chlorpromazine (*5*) or a variety of other phenothiazine derivatives (*5*, *6*) is more effective than atropine alone in preventing poisoning by sarin (*5*) or tabun (*6*).

Adult Sherman rats were used; the males weighed from 266 to 428 g and the females weighed from 175 to 300 g. Technical grade parathion, or 24.3 percent emulsifiable concentrate of Phosdrin, was diluted in peanut oil to the appropriate concentration and administered by stomach tube (5 ml/kg of body weight). Peanut oil alone was given to control rats which did not receive an organic phosphorus compound. Aqueous solutions of promazine and of chlorpromazine, 5 percent and 2.5 percent, respectively, were diluted with 0.9 percent sodium chloride solution to a concentration that permitted administration at a rate of 0.8 ml/kg of body weight. The tranquilizers were given by stomach tube or intraperitoneally. Sodium chloride was administered in like manner to control animals; atropine was given intraperitoneally. LD_{50} values were calculated by the method of Litchfield and Wilcoxon (*7*).

Groups of ten male rats were each given single oral doses of parathion. Each rat was then given 3.0 mg/kg

promazine 30 minutes after parathion, and every 6 hours thereafter until five doses had been given. To a second series of groups of ten rats, chlorpromazine was administered every 3 hours until five doses had been given. The rate of administration of these drugs corresponded to the maximum dosage given to people in cases of extreme anxiety. A third series of groups of ten rats (controls) was given a single dose of parathion followed by 0.9 percent sodium chloride solution given every 6 hours. The results are shown in Table 1.

The LD_{50} values indicated that the treatment with promazine increased the toxicity of parathion by a factor of about two and chlorpromazine increased it by a smaller factor. The LD_{50} value of parathion in the control rats corresponds closely with the value of 13.0 mg/kg for technical parathion which was reported earlier (*8*). Administration of these drugs to parathion-poisoned rats did not alter the time of onset of symptoms or the time of death.

Male rats which were given a single dose of peanut oil alone followed by dosage with promazine or chlorpromazine, as in the first and second series, exhibited marked depression but recovered overnight after treatment was discontinued (Table 1). Single doses of promazine as large as 160 mg/kg produced severe depression, from which all of the rats recovered completely.

A single 5 mg/kg dose of promazine potentiated the toxicity of 2.5 mg/kg of parathion which was given to groups of ten female rats within 30 minutes before to 1 hour after the administration of parathion. There was little effect when the promazine was given 4 hours after parathion.

Repeated dosage with promazine and atropine resulted in slightly higher mortality than atropine alone or no treatment following the same dose of parathion (Table 2). Tests with chlorpromazine gave similar results.

In all these tests, the rats that died exhibited typical symptoms of parathion poisoning.

Groups of ten female rats were each given a single oral dose of 5.0 mg/kg of promazine 30 minutes before, at the same time, and at two intervals after a single 3 mg/kg dose of Phosdrin. This dosage level of Phosdrin is slightly less than the oral LD_{50} value (3.7 mg/kg) for female rats (*8*). Promazine did not appear to have any effect upon the toxicity of Phosdrin. All of these rats exhibited typical symptoms of Phosdrin poisoning; either they died or showed a marked recovery within 2 to 3 hours. After this work was completed, we learned that Mitchell R. Zavon (*9*) had found that chlorpromazine, at dosages higher than those we had used for promazine, increased the mortality of rats previously dosed with Phosdrin (8 mg/kg). He noted the potentiation in each of three series of tests in which the rats were given repeated doses of atropine at the rate of 0, 1, and 2 mg/kg; when results of the atropine tests were combined, the mortality was 1, 7, and 15 out of groups of 15 rats which received repeated doses of chlorpromazine at the rate of 0, 5, and 10 mg/kg.

The results tentatively suggest that, in treatment of parathion or Phosdrin poisoning, tranquilizers derived from phenothiazine should either be avoided or at least used with extreme caution. We have not investigated the possibility that these drugs may increase the toxicity of other pesticides containing organic phosphorus.

Table 1. Effect of promazine (P) and chlorpromazine (CP) in male rats given repeated oral doses (3.0 mg/kg) of these compounds following a single oral dose of parathion. Promazine was given every 6 hours, chlorpromazine every 3 hours; controls were given 0.9 percent NaCl every 6 hours.

Parathion dose (mg/kg)	Mortality (No. dead/No. tested)		
	P	CP	Control
0.0	0/10	0/10	
3.0	0/10		
4.0	1/10		
5.0	7/10		0/10
6.0	5/10	2/10	
7.5		6/10	
8.0	9/10		0/10
9.0		8/10	
10.0	10/10		2/10
12.0		9/10	5/10
15.0			10/10
	LD_{50} (mg/kg)		
	5.4	7.3	12.0
	Confidence limits of LD_{50} (mg/kg)		
	4.7–6.3	5.7–9.4	10.5–13.7
	*Survival time (hr)**		
Range	1–95	2–69	1–69
Mean ± S.E.	15 ± 3.5	13 ± 2.6	14 ± 4.0

* For rats that died.

Table 2. Effect of promazine and atropine treatment in adult female rats given a single oral dose of parathion (3.6 mg/kg). Starting 30 minutes after parathion dosing, the atropine was given intraperitoneally every hour until ten doses were given and the promazine was given intraperitoneally every 3 hours until four doses were given.

Dose (mg/kg)		Onset of symptoms (hr)		Mortality (dead/tested)		Survival time (hr)	
Atropine	Promazine	Range	Mean	Test 1	Test 2	Range	Mean
0	3	1.50–2.25	1.17 ± 0.115	4/4	4/4	1–22	11
2	3	1.50–8.25	3.09 ± 0.79	2/4	4/4*	12–47	31
2	0	1.50–7.25	4.00 ± 0.96	1/4	0/4	93	93
0	0	1.25–3.25	2.03 ± 0.24	0/4	2/4	6–70	38
0	3†			0/4			

* Started treatment 30 minutes before parathion. † Peanut oil only in place of parathion.

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Simian Gastropathy With Submucosal Glands and Cysts

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Gastritis Glandularis or Cystica Profunda?

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An unusual gastropathy with submucosal glands and cysts, usually associated with mucus-secreting cell hyperplasia of the mucosa, was observed at autopsy in 11 (52%) of 21 rhesus monkeys (*Macaca mulatta*), but in none of nine squirrel monkeys (*Saimiri sciureus*). In most instances, the gastric lesion occurred spontaneously, in contrast to similar lesions in monkeys of the genus *Macaca* reported by other investigators, which usually were attributed to known exposure to irritants. Evidence in this study and in the literature suggests that the gastric mucosa of the rhesus monkey is highly reactive to nonspecific irritants. The simian gastropathy sometimes resembles an infrequently observed gastric lesion in man, reported as diffuse heterotopic cystic malformation, and is analogous to the intestinal entity, also occurring in man, known as colitis cystica profunda.

Gastric lesions characterized by prominent changes in the mucosa, including epithelial hyperplasia, and penetration of the mucosal epithelium into the submucosa, with formation of epithelial cysts, have been observed in monkeys of the genus *Macaca* (macaques) by various investigators. The lesions usually were noted in animals exposed to irritating agents (eg, polychlorinated biphenyls [PCB] or triphenyls [PCT],[1] diesel motor lubricating oil,[2,3] and an intragastric parasitic nematode, *Nochtia nochti*[4]), and only occasionally occurred spontaneously.[3]

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In the present report, similar lesions found by the author in rhesus monkeys (*Macaca mulatta*) will be described and discussed, which, however, unlike the previously reported cases, could not be related to a known cause in most instances. The apparent spontaneous occurrence of most of the lesions and the interesting features of the submucosal involvement will be emphasized. In addition, certain gastrointestinal conditions in man will be compared, including a seldom reported gastric lesion[5,6] that is morphologically similar to the simian gastropathy, at least as it appeared in some of the animals.

Materials

During a 7-month period (September 1972 through March 1973), autopsies were performed on 30 nonhuman primates, which had been involved in or were being prepared for certain research projects or were being used for breeding purposes. Of these, 21 were rhesus monkeys (*M mulatta*) and 9 were squirrel monkeys (*Saimiri sciureus*). At the time of autopsy, the estimated ages of the animals ranged from 1½ to 6 years. They had been in the laboratory for periods ranging from 2 to 55 months, being housed in harem fashion in outdoor, covered, chainlink-enclosed runs with concrete bases and no other type of bedding. The daily diet had consisted of monkey chow, supplemented by fruits two days a week, water being available through an automatic supply system. No detectable amounts of pesticides or PCB had been identified in the chow and water, as determined by periodic gas chromatographic analysis of these items.

Among the 21 rhesus monkeys, three had been subjects of experiments to determine the effects of low-level chronic exposure to pesticides administered orally, receiving respectively: DDT (0.5 mg/kg of body weight daily for 50 months), parathion (0.3 mg/kg of body weight daily for 52 months), and heptachlor (0.01 mg/kg of body weight daily for 36 months). Three of the nine squirrel monkeys had been involved in similar experiments, receiving respectively by mouth: lindane (0.03 mg/kg of body weight daily for 45 months), carbaryl (0.007 mg/kg of body weight daily for 53 months), and 2,4-D (0.2 mg/kg of body weight daily for 49 months). One rhesus monkey was given a single oral dose (13 mg) of parathion (2.9 mg/kg of body weight) one year prior to death, as part of a metabolic study. Two other monkeys (one rhesus and one squirrel) had been fed high levels of PCB in the diet. The rhesus monkey was fed PCB in the form of Aroclor 1260 in the diet in levels ranging from 500 to 2,000 parts/million/day for 47 days, followed by Aroclor 1248, 1,000 parts/million/day for 30 days. The squirrel monkey was given a daily diet containing 1,000 parts/million of Aroclor 1248 for 29 days.

Other substances that some of the animals received by mouth were vitamin preparations and anti-infectious drugs when deemed necessary. The drugs consisted of single doses of an antiparasitic agent, usually given shortly after receipt of the monkey in the laboratory, and various antibiotics, usually administered during the last week or two of life.

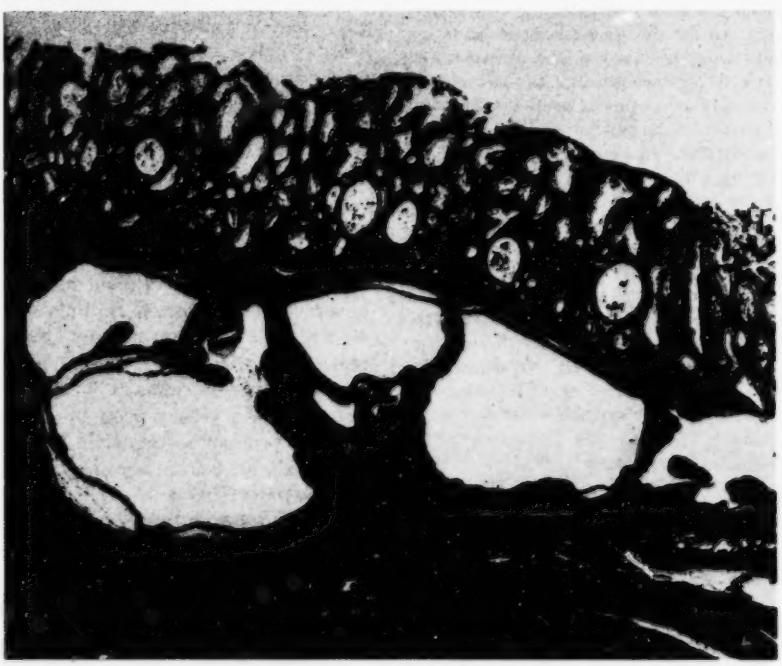
Results

An unusual gastropathy, to be described, was found in 11 of 21 rhesus monkeys. The chief clinical features observed in the affected animals were refusal to eat and loss of weight. Only one animal was eating well and gaining weight prior to death. In this instance, death was caused by fairly sudden, profuse intragastric hemorrhage resulting from erosions in the mucosa of the lesion.

Gross Description.—In all but one of the animals with the gastropathy, the lesions were diffuse, affecting a considerable portion of the stomach, but the extent of involvement varied in the individual specimens. The le-

Fig 1.—Irregular hyperplasia of gastric mucosa with penetration of muscularis mucosae. Abundant epithelium in thickened submucosa with cysts (some containing neutrophils). Smooth muscle (dark, elongated bands) in upper part of submucosa, continuous with muscularis mucosae in places (hematoxylin-eosin, original magnification × 21).

Fig 2.—Large cysts in submucosa and microcystic change in mucosa (showing mucus-secreting cell hyperplasia). Communication of submucosal cyst (right) with gastric pits in mucosa. Thin muscle bands about submucosal cysts not clearly visible at this magnification (hematoxylin-eosin, original magnification × 26).



sions were not limited to any particular anatomic area of the stomach. Generally, the mucosal surface was covered by abundant mucin. The mucosal folds appeared large in several lesions, but were small and inconspicuous in others. Hyperemia and petechiae commonly were seen in the mucosa and sometimes erosions or ulcers or both were noted.

The gastric wall in the involved area was irregularly thickened (up to 1 cm) and usually was soft or only slightly firm. On sectioning, the submucosa was especially prominent, being thickened, grayish-white, and edematous. Minute cysts were seen sometimes in the submucosa and less frequently in the mucosa. In several instances, the stomach was adherent to the liver, and when the former was pulled away, it exhibited a rough, sometimes hyperemic, serosal surface.

One of the lesions, affecting chiefly the pyloric portion but also part of the body of the stomach, was localized and extremely firm, unlike the lesions in the other animals. At the time of autopsy, it was regarded as a probable carcinoma. On sectioning, the cut surfaces were more dense than in the other lesions and it was difficult to distinguish the mucosa from the submucosa. The muscle coat, however, was readily distinguishable. The serosa over the lesion was thick, opaque, and grayish-white. The stomach was filled with abundant clotted blood and the source of bleeding was found to be several closely grouped, prominent erosions in the mucosa of the lesion.

Microscopic Description.—The lesions in the different animals were essentially similar but varied somewhat as to the amount and degree of invasiveness of the displaced tissues in the submucosa, the degree of mucosal hyperplasia or other mucosal changes, and the nature and extent of the superimposed complications.

The mucosa often was irregularly thickened, showing epithelial hyperplasia that involved particularly the mucus-secreting cells (Fig 1 and 2). Frequently, the gastric pits were considerably elongated or dilated, and sometimes formed small cysts (Fig 2).

In some areas, the mucosal pits and glands were arranged in a disorderly fashion. Occasionally, the glands in the deep portion of the mucosa were decreased in number. Foci of intestinal metaplasia were noted in a few instances. Areas of chronic inflammation were present in some specimens, being characterized by an increase in mucosal stroma and heavy infiltration by lymphocytes and histiocytes. Superimposed complications, such as petechiae, erosions, ulcers, and suppuration, were frequent. In several lesions, the mucosa appeared thin because it was partly denuded, probably as a result of inflammation or postmortem autolysis or both.

In the submucosa, which usually was considerably thickened, fibrotic, edematous, and infiltrated by mononuclear leukocytes, small nests or larger masses of epithelium were present. The epithelium in most instances was abundant or moderate in amount and often extended down to, but not through, the muscle coat (Fig 1 to 3). In several lesions, the epithelium was much less in amount and usually was limited to the upper portion of the submucosa (Fig 4). In some areas, there was continuity between the epithelium in the submucosa and that in the mucosa through defects in the muscularis mucosae (Fig 1 to 4). At several points, the muscularis mucosae showed a patchy hyperplasia, resulting in irregular thickening of this layer (Fig 1 and 3). The displaced submucosal epithelium consisted principally of tubular and glandular elements, lined by mucous columnar cells. Some of these epithelial elements were dilated and even cystic. In some cysts, the lining epithelium was undulating; in others, it was flattened and more cuboidal. The cysts varied in number and size in the different lesions, being quite prominent in some lesions and less conspicuous in others. In one specimen, the submucosal epithelium consisted almost entirely of large cysts (Fig 2). Communication between submucosal cysts and mucosal gastric pits was noted in some of the sections (Fig 2). In several lesions, particularly those with a considerable degree of proliferation of the submucosal epithelium (such as the one that was localized and simulated carcinoma on gross inspection), small nondilated glands were clustered about the cystic structures, apparently originating from outpouchings of the latter (Fig 1 and 3). The small glands often were embedded in a pale, loose, connective tissue stroma that resembled the lamina propria of the mucosa and was infiltrated by a small number of lymphocytes. Some of the cysts in the

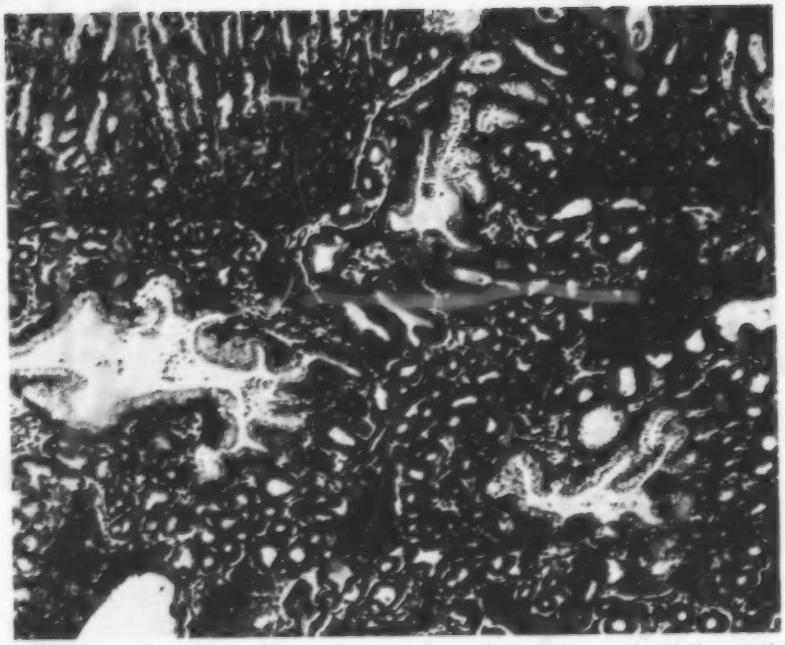
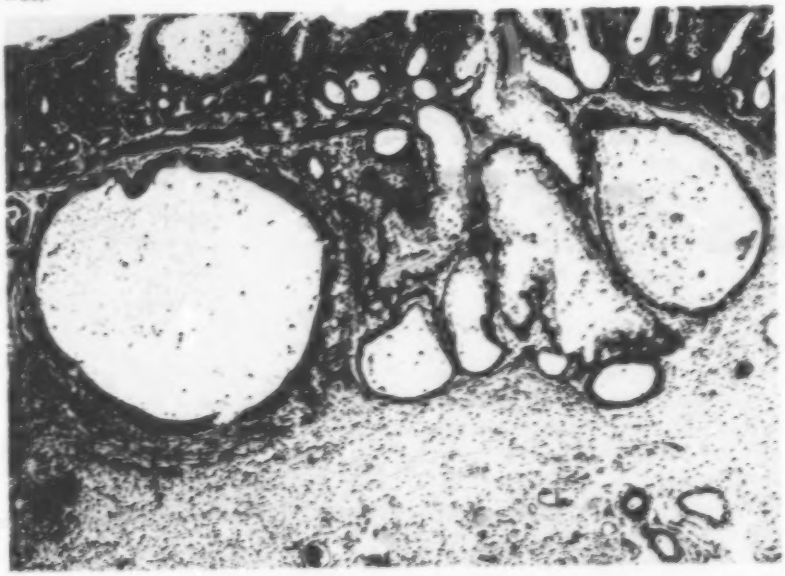


Fig 3. – Left upper quadrant of Fig 1. Note markedly proliferative submucosal epithelium, continuous with overlying mucosa, and bands of smooth muscle in submucosa (hematoxylin-eosin, original magnification × 63).

Fig 4. – Lesion showing small amount of submucosal epithelium with cyst formation, and continuity with mucosal epithelium through defect in muscularis mucosae. Thin band of smooth muscle about cyst (left). Mucosa partially denuded in this area (hematoxylin-eosin, original magnification × 56).



submucosa, as well as in the mucosa, contained neutrophils or actual suppurative exudate, which frequently extended into the adjacent stroma. Small abscesses were seen in the submucosa of several lesions.

Bands of smooth muscle often separated or surrounded masses of submucosal epithelium or individual cysts. The muscle bands were most obvious in those lesions with abundant, highly proliferative submucosal epithelium, being more prominent in the upper portion of the submucosa where some of them were seen to originate from the overlying muscularis mucosae (Fig 1). The displaced muscle was not as conspicuous in some of the lesions, particularly those with a smaller amount of submucosal epithelium (Fig 4). No parietal cells were seen in relation to the epithelium in the submucosa nor was any pancreatic tissue identified.

The muscle coat showed no obvious changes, but the serosal coat often exhibited areas of organizing fibrinous serositis or fibrous thickening. There was no evidence of a metastatic tumor in regional lymph nodes or other organs.

Occurrence.—The amount and degree of invasiveness of the submucosal epithelium were pronounced in four monkeys, moderate in three, and slight in four. Of the four monkeys with pronounced submucosal involvement, two had ingested chemical substances daily for a period of time: an approximately 4½-year-old female monkey, residing in the laboratory for 39 months and receiving low-level doses of heptachlor orally for 36 months, and a male monkey, about 2½ years old, housed in the laboratory for 13 months and being fed high levels of PCB in the diet for 77 days prior to death. The latter animal showed certain characteristic signs of PCB poisoning, such as alopecia, acneform skin lesions, hyperpigmented spots of the skin, and swelling of the eyelids associated with a thick discharge. The third monkey was an approximately 4½-year-old male, which had been in the laboratory for 38 months and received only one oral dose of parathion one year before death. The lesion in this subject was the one that was localized and simulated carcinoma grossly. It appeared to have even more proliferative submucosal epithelium than the other lesions, and was complicated by profuse bleeding that caused death. The fourth monkey was a female, about 2 years old, which had been in the laboratory for six months without experimental exposure to pesticides, PCB, or other toxic chemicals. The animals that showed moderate submucosal involvement (one male and two females) or slight submucosal involvement (one male and three females) were approximately 2 years old, had resided in the laboratory for two to six months prior to death, and had not been involved in any experiments.

Among the ten rhesus monkeys without displaced tissue in the gastric submucosa, two had slight acute gastritis in association with a bacterial gastroenterocolitis and one had gastric ulcers complicated by acute suppurative gastritis. These three animals were not subjects of any experiments. No gastric lesion was found in the other seven animals, including two that had received low-level oral doses of pesticides (DDT and parathion).

No gastric disease of any type was found in the squirrel monkeys, including the three given low-level oral doses of pesticides (lindane, carbaryl, and 2,4-D) and the one fed high levels of PCB in the diet. The PCB-treated squirrel monkey showed none of the typical signs associated with PCB poisoning, unlike the rhesus monkey exposed to PCB.

Comment

The gastropathy just described is basically a chronic nonspecific gastritis, which is characterized usually by mucosal (mucus-secreting cell) hyperplasia and complicated often by erosions, ulcers, hemorrhages, and suppuration. However, its most impressive feature is the presence of epithelium and smooth muscle in the submucosa, with formation of epithelial cysts. The histologic evidence suggests that the epithelium is derived from the mucosa, and the smooth muscle, from the muscularis mucosae, apparently as an effect of the gastritis. It is not considered likely that the displaced tissues represent a congenital malformation, such as a developmental heterotopia. The amount, degree of cystic change, and extent of involvement of the ectopic tissues vary in the different specimens, most likely in accordance with the duration of the gastropathy. At times, the submucosal epithelium is so markedly proliferative and invasive that it may lead to a mistaken diagnosis of infiltrating gastric carcinoma, an uncommon simian disease.[7,8] The smooth muscle in the submucosa is very prominent in some of the lesions, particularly those with abundant submucosal epithelium. It is probable that these are older lesions in which the displaced muscle and epithelium have had time to undergo a significant degree of hyperplasia.

Nonneoplastic invasion of the epithelium of the gastric mucosa into the submucosa, presumably the result of chronic irritation, has been described in monkeys (macaques) by several investigators. For example, the submucosal epithelium has been noted in macaques with chronic gastritis resulting from bezoars,[9] in Javanese monkeys with papillomatous proliferation of the gastric mucosa associated with an intragastric parasitic nematode, *Nochtia nochti*,[4] in rhesus monkeys with "hyperplastic gastritis" attributed to ingestion of a diesel motor lubricating oil,[2,3] and in rhesus monkeys with gastric mucosal hyperplasia ("hypertrophic gastritis") attributed to PCB or PCT in the diet.[1] In one of the reports,[2] the spontaneous occurrence of the gastric lesion was observed in two rhesus monkeys, the downward extension of the mucosa being referred to as "infiltrating hyperplastic adenoma-like lesion."

Penetration of gastric mucosal epithelium into the submucosa has been seen also in human patients with "giant hypertrophic gastritis."[10] Interestingly, displaced gastric mucosal tissue has been found in the submucosa and sometimes in the muscle and serosal coats of the stomach in mice which spontaneously develop "hyperplastic adenomatous gastritis,"[11] and

injection of a carcinogenic substance into the stomach wall in rats has been observed to induce penetration of the gastric mucosa through the muscularis mucosae, producing a lesion known as "adenomatous diverticulum."[11,12]

Of the simian gastric lesions that were mentioned previously, those observed in rhesus monkeys by Lushbaugh[2,3] and Allen and Norback[1] bear a resemblance to the gastropathy described in the present study. In their reports, cystic transformation of the epithelium in the submucosa was a prominent feature, but there was no mention of smooth muscle in association with the submucosal epithelium. A diffuse lesion, observed in two human patients by Scott and Payne[5] and Oberman et al,[6] that consisted of epithelial cysts, glands, and smooth muscle in the submucosa, as well as small cysts in the mucosa, also is very similar, resembling particularly the more proliferative lesions, such as that illustrated in Fig 1 and 3. However, the condition, named "diffuse heterotopic cystic malformation"[6] (also "diffuse congenital cystic hyperplasia"[5]) of the stomach, was interpreted by these authors as a developmental (congenital) abnormality; whereas, I consider the gastropathy in the monkeys to be related pathogenetically to gastric irritation or inflammation.

There are two other lesions involving the extramucosal layers of the stomach in man that consist of epithelium and smooth muscle, which may be considered in the differential diagnosis, although both are characteristically localized and dysontogenetic in origin. The first is a lesion originally described by Stewart and Taylor[13] as "adenomyoma" of the stomach. These authors considered it to be closely related to pancreatic heterotopia but differing from the latter in respect to the diversity of structures present, namely, glandular tissue of gastrointestinal (secretory) type, undifferentiated duct-like tissue, and smooth muscle, in addition to pancreatic acini. The lesion may be found in both the submucosa and muscle coat or in the muscle coat alone. The second lesion, termed "myoepithelial hamartoma" by Clarke,[14] consists of smooth muscle, duct-like structures, and Brunner-like glands, with or without pancreatic tissue, and is noted usually in the gastric submucosa but sometimes in the muscle and serosal coats. Although there is some similarity, morphologically, between the submucosal involvement in the stomachs of the monkeys in the present study and these two gastric lesions in man, particularly the myoepithelial hamartoma, certain differences are apparent, as evidenced by the following features observed in the simian lesions: (1) With one exception, the lesions tended to be diffuse. (2) Pancreatic tissue was not present in any of the lesions. (3) Communication was demonstrated between some of the submucosal cysts and gastric pits in the mucosa. (4) Usually, some degree of hyperplasia with cystic change was noted in the overlying mucosa. (5) No penetration of the muscle or serosal coats by the displaced tissues was evident.

Rarely, a single intramural mucin-containing epithelial cyst, with a layer of smooth muscle around it, is seen in the stomach of man, which is described as an "accessory cavity or reduplication."[15]

The presence of submucosal cysts in the simian gastropathy, particularly in those instances in which the cysts are prominent (as illustrated in Fig 2), is somewhat analogous to what is seen in an unusual disease involving the large intestine in man, known as "colitis cystica profunda."[16-18] This lesion is characterized by mucosal glands that extend down into the submucosa and become hyperplastic and cystic. It is interesting that in one series of cases, smooth muscle originating from the muscularis mucosae was found adjacent to the cysts in the colonic submucosa,[18] a finding which is similar to that seen in the simian gastric lesions. Whether colitis cystica profunda is a congenital or acquired disease is debated by some investigators, but the prevalent view seems to be that it is postinflammatory. Because of a certain similarity between this intestinal lesion and the simian gastropathy, perhaps the designation, "gastritis cystica profunda," for the latter would be appropriate. However, in some of the simian lesions, the glandular elements appear to be more prominent than the cysts, so that terms, such as "gastritis glandularis profunda" or "gastritis glandularis and cystica profunda," may be suggested. In a recent article, a polypoid gastric lesion occurring at a gastroenterostomy site, with hyperplastic and cystic mucosal epithelium infiltrating the submucosa, was named "gastritis cystica polyposa."[19]

An observation that is worthy of note is that two rhesus monkeys in this study, which had not been involved in any experiments, showed colonic lesions characterized by penetration of mucosal glands into the submucosa, with cystic transformation. In one of the animals, gastropathy with submucosal cysts also was present (Fig 4). The other animal did not have associated gastric disease. The colonic mucosa and submucosal cysts contained neutrophils in the first instance and abscesses in the second, apparently as a result of bacterial infection. Lushbaugh described similar intestinal lesions in rhesus monkeys ingesting diesel motor lubricating oil.[3] This intestinal involvement in the monkeys deserves further study.

Regarding the cause of the simian gastropathy, no single factor was found to account for all of the lesions. In three instances, PCB, heptachlor, and parathion, each administered orally to a different animal with the gastric disease, could be considered as possible causative factors, particularly PCB and heptachlor, which were given repeatedly over a period of time. However, it is rather difficult to determine the actual role of parathion since only one oral dose of this pesticide was administered.

The other animals with the gastropathy were not experimentally exposed to PCB, pesticides, or other chemicals. Their gastric lesions seemed to have occurred spontaneously without a demonstrable cause. It is doubtful that the orally administered vitamins or anti-infectious drugs played a role in the origin of the lesions. The antibiotics usually

were given shortly before death when the lesions undoubtedly were already present. The single therapeutic dose of an antiparasitic drug was given to less than half of the rhesus monkeys with the gastropathy, as well as to about half of those without this condition. Infectious agents and hypersensitivity may be considered as possible etiologic factors; but, unfortunately, immunologic and systematic microbiologic investigations were not carried out in this study. There was, however, no evidence of worms or other parasites in or near the lesions, either on gross inspection or microscopically. The one disease that was common among the rhesus monkeys with the gastropathy was lung-mite infection, but this disease was also prevalent among the rhesus monkeys without gastric lesions. It is difficult to relate this infection to the gastropathy, unless one speculates that certain irritant substances derived from the mites or the resultant pulmonary lesions might be involved in some way, after being coughed up and swallowed. It should be noted that lung mites are commonly found in Old World monkeys (eg, the macaques, including rhesus monkeys) but are hardly ever observed in New World monkeys (eg, the squirrel monkeys). There was no evidence of this parasite in any of the squirrel monkeys in this study.

A factor that can not be adequately assessed, but one that may be considered in regards to the pathogenesis of the gastric lesions, is psychologic stress, such as that which may occur as a result of captivity, shipment, and frequent handling. It is generally known that gastric erosions, ulcers, and hemorrhages can occur in nonhuman primates subjected to various psychologic stresses.[20] However, it is not certain whether repeated stressful situations are influential in the development of chronic gastritis. Also, it can not be determined whether any possible factors, such as nutritional deficiency or irritant substances, were involved prior to captivity.

A significant observation in this study is that none of the squirrel monkeys, including those ingesting pesticides or PCB, developed the gastropathy that was so common in the rhesus monkeys. This may be an indication that the rhesus monkey is more susceptible to gastric irritants than the squirrel monkey, which is a more primitive form of primate than the rhesus, but more study is needed to verify this. It is probable, as pointed out by Lushbaugh,[3] that the gastric mucosa, as well as the colonic mucosa, of the rhesus monkey is extremely reactive to nonspecific irritants, and this also may be true for the other members of the genus *Macaca*. It would be of interest to investigate whether the gastric lesions observed in the macaques also occur in those nonhuman primates that are higher in the evolutionary scale, such as gibbons, orangutans, chimpanzees, and gorillas.

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The Toxicity of Amitrole in the Rat

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The Toxicity of Amitrole in the Rat. GAINES, T. B., KIMBROUGH, R. D. AND LINDER, R. E. (1973). *Toxicol. Appl. Pharmacol.* **26**, 118–129. A single oral dose of 4080 mg/kg and a single dermal dose of 2500 mg/kg of amitrole, the highest doses given, were not lethal to adult rats. Dietary levels of 0, 1000, 500, 100 and 25 ppm amitrole were fed to groups of 10 male and 10 or 12 female rats. The dietary levels of 1000 and 500 ppm were fed for 107–110 days, the dietary levels of 100 and 25 ppm for 240–247 days to the F_0 generation and for 141 days to the F_{1b} generation. At the dietary levels of 1000 and 500 ppm the pups that were born were smaller and had atropic thymuses and spleens indicative of runt disease. Most of them had died within a week after weaning. Reproduction was not affected at the lower dietary levels. Hyperplasia of the thyroid was seen in all rats fed dietary levels of 100 ppm or greater. In the 25 ppm group 4/10 F_0 and 4/10 F_{1b} females and 6/10 F_{1b} males, but none of the F_0 males developed hyperplasia of the thyroid.

The herbicide amitrole (3-amino-1,2,4-triazole) is used as a spot treatment for weed control in industrial and other noncrop areas. It cannot legally be used on crops or pasture land where food for man or animals may become contaminated.

Weir *et al.* (1958) reported that dietary levels as high as 10 ppm amitrole had no effect on growth, on thyroid activity, or on other organs, but 50–100 ppm had minimal effect on the thyroid as manifested by hyperplasia of this gland. Jukes and Shaffer (1960) found that amitrole caused adenomatous changes in the thyroid glands of rats fed 100 and 500 ppm amitrole for 24 months. Such changes were found to be reversible when amitrole was withdrawn. Kröller (1966) in an excellent paper, reviewed the toxicity, chemistry, and application of amitrole.

A 39-year-old woman who ingested 20 mg/kg of amitrole and excreted 100 mg/100 ml of urine some hours later showed no signs of intoxication (Geldmacher-Von Mallinckrodt and Schmidt, 1970). Landauer *et al.* (1971) reported that amitrole injected into chicken eggs at a level of 20 and 40 mg per egg caused a high incidence of malformations, principally of the beak with abnormalities of the tibia shaft as a less common defect.

Few data on the toxicity of amitrole are available in the published literature and information on its effect on reproduction is lacking. It is the purpose of this paper to report the results of our studies on the oral toxicity of amitrole, including its effect on reproduction in rats. Most of the study was done before the more recent restriction was placed on the use of the compound.

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METHODS

All tests were conducted with the same batch of 99% pure amitrole. The rats were Sherman strain produced under specific pathogen-free conditions.

Acute oral toxicity studies. Ten male and 10 female, 5-month-old rats were dosed by stomach tube with 24% amitrole in water solution given at the rate of 17 ml per kg of body weight (4080 mg/kg of amitrole). Dosing was done according to the method described by Gaines (1960). The male rats weighed 483–590 g and the females 303–323 g.

In acute dermal toxicity studies a 25% solution of amitrole in water was applied as a single dose to the skin of 10 male and 10 female 6-month-old rats at the rate of 10 ml/kg of body weight (2500 mg/kg of amitrole). The rats were prepared for dermal dosing by clipping the hair. The males weighed 451–605 g and the females 225–323 g. Surviving rats were observed for a minimum of 14 days.

Reproduction studies. Five- to 6-week-old rats were fed amitrole in the diet. The diets were prepared by mixing amitrole with ground laboratory chow according to the procedure described by Gaines and Kimbrough (1968). The rats were individually caged except during pair-mating and were fed ad libitum including the period of gestation and lactation. The F_0 generation rats were weighed weekly and observed for signs of toxicity. Rats from subsequent generations used for breeding stock were checked closely each week for signs of toxicity but were weighed only at the time of selection (4–5 weeks old) just prior to pair-mating and when killed. All litters were weaned when 21 days old.

Animal tissue for light microscopic examination was fixed in 10% buffered formalin and stained with hematoxylin and eosin. Tissue for the electron microscope was fixed in 5% phosphate-buffered glutaraldehyde for 3 hr, postfixed in 1% osmium tetroxide, cut with a glass knife and stained with a combination of lead citrate and uranyl acetate or with lead citrate alone.

In a *preliminary reproduction* study groups of 10 male and 10 female rats each were fed amitrole at dietary levels of 0 (control), 500 and 1000 ppm, respectively, for 55 days and then pair-mated. Food consumption was measured daily during the second, third and eighth weeks. The offspring were weaned at 21 days and held for observation. After weaning of the litters, the parent rats were killed after a total exposure of 107 and 108 days for the males to 110 days for the females. Terminal hematocrit, white blood cell counts (WBC) and differential values were determined. Complete autopsies were performed and the weights of the heart, kidneys, liver, lungs, spleen and testes were recorded. These and other selected tissues, including the thyroid were fixed for examination with the light microscope. Ten F_1 weanling rats each from the control group and the 500 ppm group were killed and the thymus and spleen removed for study with the light microscope.

Because of the effect of amitrole on reproduction in the preliminary study groups of 10 male and 10 female rats each were fed amitrole at dietary levels of 0 (control), 25 and 100 ppm, respectively. Food consumption was measured daily during weeks 2, 4, 8, 18 and 23. The rats were pair-mated after 61 and 173 days to produce the F_{1a} and F_{1b} generations, respectively. The thymus and spleen from 8 weanling rats of each sex in the 100 ppm and control groups of the F_{1a} generation were examined microscopically. Breeding stock (12 males and 12 females from each dietary level of amitrole) was selected from the F_{1b} rats, continued on the same diets as the F_0, and then pair-mated when about

100 days old to produce the F_{2a} generation. All the F_{1a} and F_{2a} and all the F_{1b} rats, except those saved for breeding stock, were killed at weaning. After weaning of the F_{1b} rats, the F_0 rats were killed and after weaning of the F_{2a} rats, the F_{1b} breeding stock was killed. Terminal WBC, hematocrit, hemoglobin and differential cell counts were determined. The brain, heart, kidneys, liver, lungs and spleen were weighed. These organs, the thyroid and parathyroid from the F_0 rats were examined by light microscope. Only the thyroid and kidneys from the F_{1b} rats were examined microscopically. Thyroid tissue from 3 F_{1b} female rats each from the control group and the 100 ppm group were examined with the Philips EM 300 electron microscope.

The effect of *oral intubation* of amitrole upon reproduction was also studied in 3 groups of 8 pregnant female rats given amitrole by stomach tube at dosage levels of 0 (control), 20 and 100 mg/kg/day, respectively. The amitrole was dissolved in tap water in the appropriate concentrations to give the indicated dosage levels by administering the solution at a constant volume of 5 ml/kg of body weight. The controls were given tap water at the same rate. The date of insemination of the rats was determined by daily microscopic examination of a vaginal smear from each female during pair-mating. The day when sperm was observed in the smear, was counted as day 0. The amitrole was given daily on day 7 through day 15 of pregnancy (a total of 9 consecutive doses). The rats were allowed to litter, and the pups were weaned and discarded when 21 days old. The pups were observed for gross abnormalities and weighed at birth. They were checked daily for mortality during lactation, weighed and checked closely for abnormalities when weaned. The Chi square test and the Student *t* test were used for statistical evaluations.

RESULTS

No signs of toxicity by clinical observation were seen in adult male or female rats given 4080 mg/kg of amitrole in a single dose by stomach tube or in adult rats given 2500 mg/kg by the dermal route. These were the highest single dosage levels tested.

None of the rats fed amitrole in the diet died, except offspring of those fed 500 and 1000 ppm in the preliminary study. None of the rats fed 25 or 100 ppm exhibited signs of toxicity. Of the parent stock fed 500 and 1000 ppm amitrole, 4 rats showed some mild respiratory distress and 1 developed an adenocarcinoma of the mammary gland. Adult male rats fed 5000 ppm in the diet (about 150 mg/kg/day of amitrole) for 90 days showed no gross signs of toxicity except for a weight loss of about 15% during the first 6 weeks of exposure (unpublished data from our laboratory).

The effect of amitrole in the diet upon body weight gain and incidence of hyperplasia of the thyroid in rats is shown in Table 1. Rats fed 500 and 1000 ppm gained 14 to 26% less body weight than did the controls. The treated females consumed about 10% less and the treated males about 5% less food than the corresponding controls. F_0 generation rats in the 100-ppm group gained as much weight as the controls, although the males consumed 8.5% less food and the females 6.4% less than the controls. Food consumption was not reduced in rats fed 25 ppm.

Hyperplasia of the thyroid was seen in all rats fed dietary levels of 100 ppm or greater of amitrole. About half of the F_0 (4/10) and F_{1b} (4/10) females and F_{1b} (6/10) males, but none of the F_0 males, in the 25-ppm group developed hyperplasia.

TABLE 1

BODY WEIGHT GAIN AND THYROID HYPERPLASIA IN RATS FED AMITROLE IN THEIR DIET

Generation	Sex	Days on diet	Amitrole		Mean body weight gain (%)	Incidence of hyperplasia of thyroid[b]
			Dietary level (ppm)	Dosage level (mg/kg/day)[a]		
F_0[c]	F	110	0	0	94	0
			500	43–31	79	10
			1000	86–68	79	10
F_0[c]	M	107	0	0	144	0
			500	42–28	118	10
			1000	87–60	124	10
F_0	F	240	0	0	170	0
			25	2.5–1.3	168	4
			100	9.6–4.8	165	10
F_0	M	247	0	0	278	0
			25	2.5–1.1	285	0
			100	9.5–4.2	286	10
F_{1b}	F	141[d]	0	—	443	0
			25	—	512	4
			100	—	461	9
F_{1b}	M	141[d]	0	—	749	0
			25	—	811	6
			100	—	735	10

[a] Food consumption (g/kg/day) diminished with maturity, and this in turn decreased the daily amitrole intake (mg/kg/day).

[b] Values based on 10 rats of each sex at each dietary level, except for only 9 females in the 100-ppm group in the F_{1b} generation.

[c] F_0 in preliminary study.

[d] Time from weaning to sacrifice.

The terminal hemoglobin, hematocrit, WBC, and differential values for rats fed amitrole in the diet did not differ from the values of the control groups.

Relative weights (organ weight/body weight × 100) of kidneys, liver, and spleen of rats fed amitrole in the diet are shown in Table 2. The rats were all used in reproduction studies. Those in the preliminary study fed 500 and 1000 ppm amitrole and the F_{1b} in the 25- and 100-ppm groups were pair-mated once. The F_0 rats in the 25- and 100-ppm groups were pair-mated twice (after 61 and 173 days, respectively). The kidneys of all rats, except for the F_0 females in the 500-ppm group and the F_{1b} males and females in the 25-ppm group were significantly smaller than the kidneys of the controls ($p<0.05$). The livers of the males in the 500- and 1000-ppm groups, the F_{1b} males in the 25- and 100-ppm groups, and the F_{1b} females in the 100-ppm group were significantly smaller than the livers of the controls. Only the males and females in the 500- and 1000-ppm groups and the F_{1b} females in the 100 ppm group showed a significant decrease in

TABLE 2

RELATIVE WEIGHTS OF KIDNEYS, LIVER AND SPLEEN OF RATS FED AMITROLE IN THE DIET

Dietary level (ppm)	Sex	Generation	Organ weight as percent of body weight[a]					
			Kidney		Liver		Spleen	
			Mean ± SE	p	Mean ± SE	p	Mean ± SE	p
0	M	F_0[b]	0.62 ± 0.02	—	2.80 ± 0.07	—	0.15 ± 0.005	—
500	M		0.53 ± 0.01	<0.01	2.51 ± 0.06	<0.01	0.12 ± 0.008	<0.01
1000	M		0.54 ± 0.01	<0.01	2.51 ± 0.09	<0.05	0.13 ± 0.005	<0.01
0	F	F_0[b]	0.67 ± 0.02	—	3.32 ± 0.12	—	0.19 ± 0.010	—
500	F		0.62 ± 0.02	NS[a]	3.38 ± 0.17	NS	0.16 ± 0.004	<0.02
1000	F		0.62 ± 0.01	<0.05	3.65 ± 0.13	NS	0.16 ± 0.005	<0.05
0	M	F_0	0.55 ± 0.02	—	2.62 ± 0.06	—	0.14 ± 0.008	—
25	M		—[c]		—[c]		—[c]	
100	M		0.51 ± 0.01	<0.05	2.64 — 0.06	NS	0.13 ± 0.008	NS
0	F	F_0	0.63 ± 0.01	—	3.05 ± 0.10	—	0.18 ± 0.008	—
25	F		0.59 ± 0.02	<0.05	—[c]		—[c]	—
100	F		0.56 ± 0.02	<0.005	3.08 ± 0.12	NS	0.16 ± 0.011	NS
0	M	F_{1b}	0.53 ± 0.01	—	2.73 ± 0.10	—	0.15 ± 0.006	—
25	M		0.51 ± 0.03	NS	2.38 ± 0.08	<0.025	0.16 ± 0.007	NS
100	M		0.49 ± 0.01	<0.05	2.48 ± 0.05	<0.05	0.14 ± 0.005	NS
0	F	F_{1b}	0.64 ± 0.01	—	3.31 ± 0.11	—	0.19 ± 0.008	—
25	F		0.64 ± 0.01	NS	3.24 ± 0.07	NS	0.19 ± 0.009	NS
100	F		0.51 ± 0.01	<0.001	2.97 ± 0.11	<0.05	0.16 ± 0.005	<0.010

[a] Each value is based on data of 10 animals. Probability values were derived using Student's t test; comparisons were made with control values; p values larger than 0.05 were considered not significant (NS).

[b] Preliminary feeding study; males were killed 107 and 108 days after onset of exposure and females were killed 110 days after onset of exposure.

[c] Organ weights were not recorded.

spleen weight. The weights of the brain, heart, lungs and testes (not shown in Table 2), of the treated rats did not differ from the weights of these organs in the control rats. The thyroid of the rats fed 100, 500 and 1000 ppm amitrole were noticeably enlarged at autopsy. They were not weighed because better sections for microscopic study are obtained when they are left attached to the trachea.

Microscopically the enlarged thyroids (Figs. 1 and 2) showed smaller than normal follicles with very little pale colloid. The epithelium was mostly high columnar; small adenomas were observed in 4 of 10 rats fed 1000 ppm amitrole. Occasionally infiltration of the capsule of the thyroid by lymphocytes was also observed in the rats that had enlarged thyroids. Examination of normal and hyperplastic thyroid tissue with the

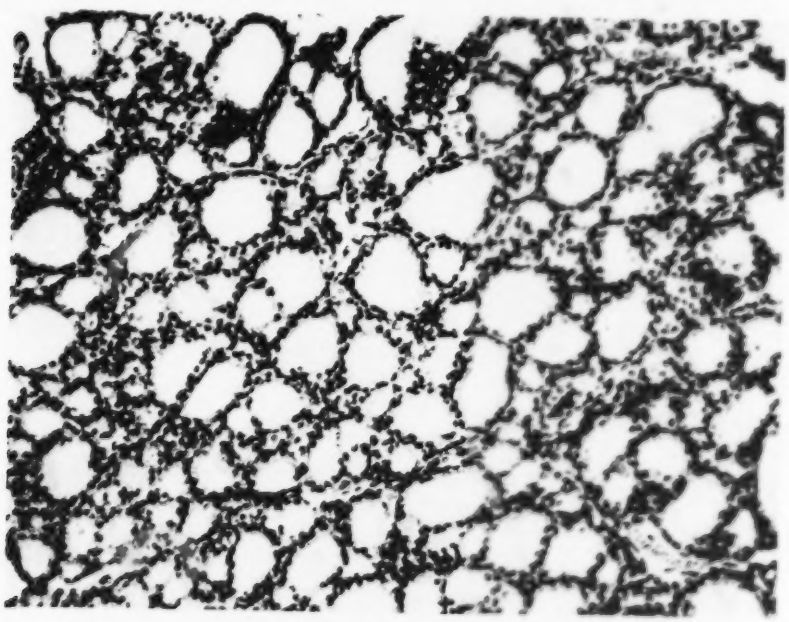

FIG. 1. Normal thyroid from control rat. Hematoxylin and eosin stain, × 100.

electron microscope showed (Figs. 3 and 4) no striking difference between the epithelium of the hyperplastic and normal thyroid. The cytoplasm of the follicular epithelium in normal thyroid tissue has lacunae which vary greatly in size and contain a granular lightly staining material when stained with lead citrate and uranyl acetate. The epithelium lines follicles of varying sizes. The colloid in the follicles is also granular but more dense than the material observed in the lacunae of the follicular epithelium. The apical borders of the follicular cells are provided with microvilli which vary greatly in numbers. A very detailed description of the fine structure of the thyroid gland in the rat is given by Wissig (1960). The follicular epithelial cells of the hyperplastic thyroids had lacunae containing a granular material similar to those observed in the cytoplasm of the controls. However, the great variation in the size of the lacunae was not evident. The follicles were smaller but contained also a dense granular material. Dense round bodies were

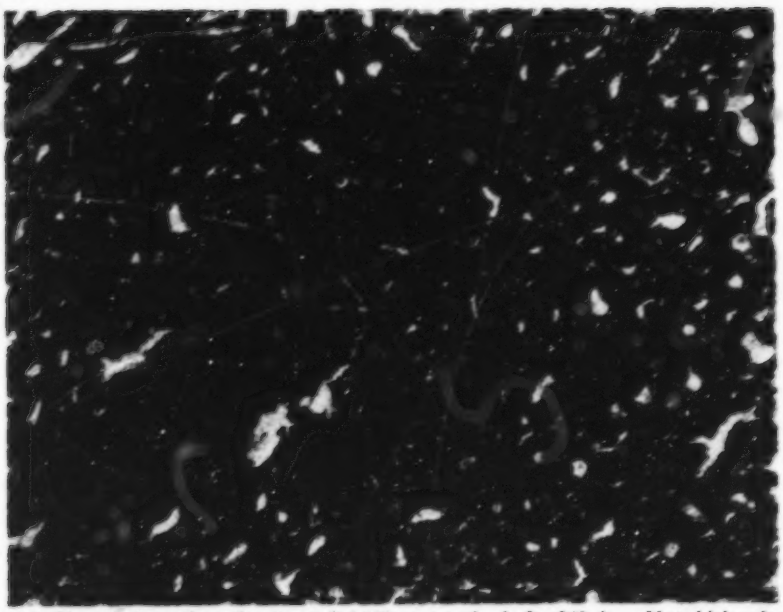

FIG. 2. Thyroid tissue from female rat fed 100 ppm amitrole for 240 days. Note high columnar epithelial cells and small follicles. H & E stain, × 100.

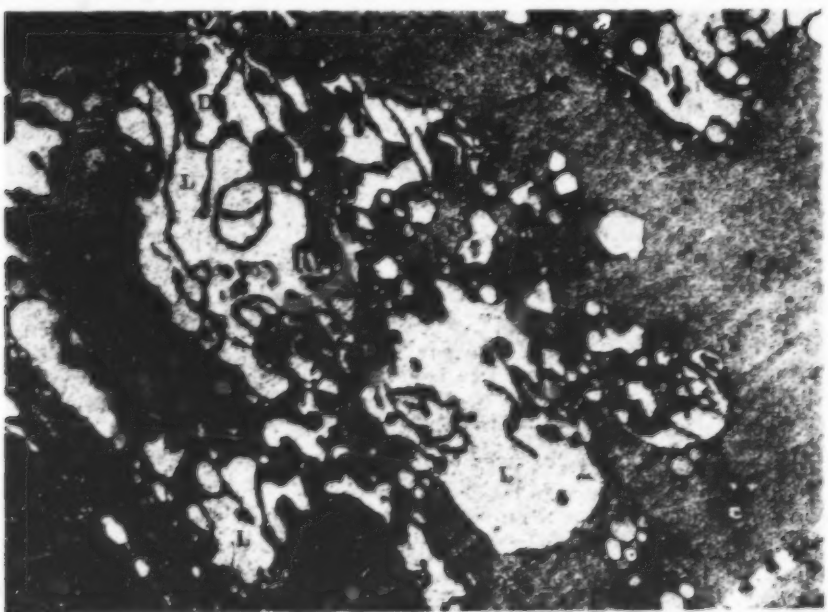

FIG. 3. Electron micrograph of normal thyroid tissue. N = nucleus, L = lacunae, F = follicle, MV = microvilli, DB = dense bodies. Note the granular material within the lacunae and the follicles. Lead citrate and uranyl acetate stain, × 8975.

observed in the cytoplasm of follicular cells from controls and from rats exposed to amitrole. These dense bodies were more frequent in the control rats.

Occasional rats in our colony develop chronic pyelonephritis, particularly with increasing age. Kidney disease was also observed in the present study. Amitrole did not affect the spontaneous incidence of kidney disease in the study since the incidence in the controls was about the same.

FIG. 4. Electron micrograph from hyperplastic thyroid of rat fed amitrole. N = nucleus, DB = dense bodies, L = lacunae, BV = blood vessel, CB = cell border, M = mitochondria. Note the relative uniformity of the lacunae within the cytoplasm of the follicular epithelium. Lead citrate and uranyl acetate stain × 8975.

The results of reproduction studies in the rats fed amitrole in the diet are summarized in Table 3. The number of pups born per litter and the mean body weight of the pups at weaning were reduced in the 500- and 1000-ppm groups. The percent survival of the pups to weaning was also reduced in the 1000-ppm group compared with survival of the controls. The pups were weak, small and generally stunted. In fact, 55 of 56 pups from dams fed 1000 ppm amitrole and 33 of 45 pups from dams fed 500 ppm died within a week after weaning. None of the controls died. Ten pups each from the control group and the 500-ppm group were sacrificed when 24 to 26 days old and the thymus and spleen taken for examination with the light microscope. Based on relative weight (organ weight as percent of body weight), these organs from the 500-ppm group were significantly smaller than those from the control group ($p<0.001$). The mean relative weight $\pm$ SE of the spleens of the 500-ppm group pups was 0.17 ± 0.01 g compared with 0.35 ± 0.02 g in the control group. These values for the thymus were 0.11 ± 0.01 g in the

TABLE 3

REPRODUCTION AND SURVIVAL OF PUPS FROM RATS FED AMITROLE IN THEIR DIET

Generation	Dietary level (ppm)	Days on diet[a]	No. of litters		Average No. per litter				Survival to weaning (%)	Mean body wt at weaning (g)
			Born[b]	Weaned	Born Alive	Male	Weaned Female	Total		
F_1[c]	0	55	6	6	12.2	5.3	6.0	11.3	93.1	37.4
	500		6	6	8.5	4.5	3.0	7.5	88.2[d]	30.7
	1000		8	8	9.1	3.1	3.9	7.0	76.7[d]	30.1
F_{1a}	0	61	8	8	11.4	6.0	4.9	10.9	95.6	42.9
	25		8	8	12.1	5.8	5.8	11.6	94.8	41.8
	100		9	9	12.2	5.7	6.4	12.1	99.1	39.0
F_{1b}	0	173	9	9	10.9	5.1	5.4	10.5	96.9	42.2
	25		5	5	11.8	7.6	4.2	11.8	100.0	39.0
	100		7	7	11.7	5.9	5.6	11.5	97.5	35.4
F_{2a}	0	108	11	11	10.9	4.5	6.1	10.6	97.5	41.0
	25		12	12	12.6	6.0	6.1	12.1	96.0	37.7
	100		9	9	9.9	5.1	4.6	9.7	97.8	43.6

[a] At time of pair-mating, the rats were continued on respective diets during mating, gestation and lactation.
[b] Ten pair-matings at each dietary level for the F_1, F_{1a} and F_{1b} generations and 12 pair-matings at each dietary level for the F_{2a} generation.
[c] Preliminary feeding study.
[d] 55 of 56 pups from dams fed 1000 ppm and 33 of 45 pups from dams fed 500 ppm amitrole died within a week after weaning (no controls died).

TABLE 4

REPRODUCTION IN RATS DOSED BY STOMACH TUBE WITH AMITROLE FROM DAY 7 THROUGH DAY 15 OF PREGNANCY

Dosage level (mg/kg/day)	No. of females Bred[a]	No. of litters		Average No. per litter					Survival to weaning (%)	Mean body wt (g)
				Born		Weaned				
		Born	Weaned	Alive	Dead	Male	Female	Total		
0	8	8	8	12.1	0	6.1	6.0	12.1	100.0	35.9
20	8	8	8	12.6	0	6.5	5.6	12.1	96.0	34.7
100	8	8	8	10.9	0	4.8	6.1	10.9	100.0	37.4

[a] Verified by microscopic examination of a vaginal smear.

500-ppm group and 0.33 ± 0.02 in the controls. Microscopically the lymphocytes in all the thymuses and in half of the spleens appeared reduced and many had pycnotic nuclei.

Compared with the corresponding control groups, there was a very slight decrease in the weight of the weanling rats in the F_{1a} and F_{1b} generations of the 25- and 100-ppm groups (Table 3). Fewer litters were born to the 25-ppm group in the F_{1b} and to the 100-ppm group in the F_{2a}. The litters also had slightly fewer pups in the F_{2a} generation of the 100-ppm group. However, because of the variation among the 25- and 100-ppm groups in the different generations, there does not appear to be any effect on reproduction in these groups that is clearly associated with dietary level of amitrole and the difference was not significant on the basis of the Chi square test. No gross physical abnormalities were observed in any of the rats. The thymuses and spleens from 8 male and 8 female weanling rats of the 25- and 100-ppm groups were normal in size. No structural changes were detected by examination of these organs with the light microscope.

The data in Table 4 indicate that there was no effect of amitrole upon reproduction in rats when the compound was given to pregnant females at levels of 20 and 100 mg/kg/-day from day 7 through day 15 of pregnancy. No abnormalities were observed in the offspring at the time of birth or when weaned.

DISCUSSION

The antithyroid compounds all produce a decrease of circulating thyroid hormone which stimulates the pituitary gland to produce an excess of thyrotropic hormone that causes hyperplasia of the thyroid gland. Amitrole inhibits thyroid peroxidase (Strum & and Karnovsky, 1971). Thyroid peroxidase converts iodide to an "oxidized iodide" form which iodinates the tyrosine residue of thyroglobulin.

The results of our study on the toxicity of amitrole are comparable to those reported earlier (Kröller, 1966). A dietary intake of 25 ppm produced hyperplasia of the thyroid in some female rats in our study. The lowest dietary level to have this effect reported previously was 50 ppm (Kröller, 1966; Weir, 1958). The longest exposure time to amitrole in our rats was about 8 months. No evidence of carcinoma of the thyroid was found during that time. Jukes and Shaffer (1960) found changes of the thyroid interpreted as adenocarcinoma in rats that were fed 100 ppm amitrole for 2 years. Metastases were not observed. It is possible that the thyroid tumor production is due to the increased thyrotropic hormone which has in itself a carcinogenic effect (Sinha *et al.*, 1965).

Amitrole has a teratogenic effect in chick embryos (Landauer *et al.*, 1971). The principal malformation was observed in the beak and abnormalities of the tibia occurred less often. Malformations were not observed in the offspring of our reproduction study, but a marked effect on the survival of the pups was noticed when 1000 or 500 ppm amitrole was fed to the dams. Both of these dietary levels resulted in runt disease of the pups with atrophy of the thymus and spleen. Our study did not resolve the question whether the production of runt disease was caused by transplacental passage of amitrole or by its excretion in the milk of the dams or even the contaminated diets to which the pups were exposed. Since at the dietary levels of 500 and 1000 ppm the number of pups born per litter was reduced, transplacental passage of amitrole is probably a contributing factor.

Electron microscopic examination shows that the size of the lacunae in hyperplastic

thyroids is smaller which is consistent with the fact that less thyroid hormone is produced. The marked increase in vascularity in the thyroids observed by Strum and Karnovsky (1971) was not evident in our study. In general, examination of the hyperplastic thyroid with the electron microscope seemed to indicate a different functional state of the cell but no other alterations.

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HEPATIC PORPHYRIA INDUCED BY 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IN THE MOUSE

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ABSTRACT

Oral administration of 4 weekly doses of 25 µg/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin resulted in induction of δ-aminolevulinic acid synthetase and hepatic porphyria in mice. There was a 2,000-fold increase in the liver content of 8- and 7-carboxyporphyrins. A single lethal oral dose of 150 µg/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin also resulted in a 4,000-fold increase in uroporphyrins in the liver. Doses of tetrachlorodibenzo-p-dioxin which resulted in porphyria also resulted in microscopic evidence of marked liver damage and a moderate increase in the total iron content of the liver. At this time, 2,3,7,8-tetrachlorodibenzo-p-dioxin, a contaminant of a variety of environmental chemicals, is the most potent porphyrogenic chemical known.

INTRODUCTION

The chlorinated dibenzofurans and dibenzodioxins are contaminants of a variety of environmental chemicals, and at least some are potent hepatotoxic, chloracneogenic, and teratogenic agents (Vos et al., 1970; Schwetz et al., 1973; Bauer et al., 1961; Linn Jones and Krizek, 1962). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a contaminant of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), causes lethal liver necrosis at doses of 100 µg/kg in

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the rabbit (Bauer et al., 1961; Schwetz et al., 1973). Tetrachlorodibenzofuran is a contaminant of some commercial preparations of polychlorinated biphenyls (PCB's), another environmental contaminant (Vos et al., 1970).

PCB's, chlorinated benzenes, chlorinated phenols, and 2,4,5-T have been reported to produce hepatic porphyria (Ockner and Schmid, 1961; Rimington and Ziegler, 1963; Bleiberg et al., 1964; Vos et al., 1970, 1971). Recently, Poland and Glover (1973) have shown that several chlorinated dibenzodioxins induce hepatic δ-aminolevulinic acid (ALA) synthetase, the rate-limiting enzyme in porphyrin and heme synthesis, in the chick embryo. TCDD was the most potent of the chlorinated dibenzodioxins (3 ng/kg). The potency of the compound in this system raises the possibility that even low levels of TCDD as a contaminant of chlorinated phenol products could result in porphyria. However, a number of compounds which are porphyrogenic in the chick (chlordane, DDT, and steroids) are not porphyrogenic in mammals (Granick, 1966), and in a preliminary study, Woods (1973) reported that administration of 5, 25 or 100 μg/kg TCDD did not induce ALA synthetase or cause accumulation of porphyrins in the liver as indicated by fluorescence at periods up to 30 days after oral administration to male rats. Similar observations were made after treatment of mice and guinea pigs with TCDD.

In the present study, mice were dosed with single and multiple doses of TCDD and, after 28 days, livers were analyzed for ALA synthetase activity and for porphyrins by thin layer chromatography.

METHODS AND MATERIALS

Fifty-four male C57BL/6 mice (29-34 days old, mean weight 18.1 g, ranging from 16.0 to 23.6 g) were divided into 3 groups of 12 each and 1 group of 18. Mice were dosed orally with 4 weekly doses of 0, 1, 5 or 25 μg/kg TCDD

dissolved in acetone and subsequently diluted with at least 6 parts of corn oil. The group receiving the highest dose contained 18 animals. Animals were killed 7 days after the last dose. Livers were removed and a portion was homogenized in 0.9% NaCl containing 0.5 mM EDTA and 10 mM tris buffer for the assay of ALA synthetase. The remainder was frozen for analysis of porphyrins. ALA synthetase was assayed in whole liver homogenates by the method of Gross and Hutton (1971), and ALA was estimated after column chromatography (Marver et al., 1966b). Nine of the 18 mice receiving 25 µg/kg TCDD for 4 weeks died 2-3 days before sacrifice, and their livers were obtained after death for analysis of porphyrins. A second group of 29 mice (3 months old) given a single oral dose of 150 µg/kg TCDD died or were killed moribund 17-30 days after treatment (93% mortality). Livers of mice which died or were killed moribund 21-25 days after treatment were removed for analysis of porphyrins.

Porphyrins were extracted, separated by thin-layer chromatography (Doss, 1967) and estimated by spectrophotometry with a Cary 14 spectrophotometer (Dresel and Falk, 1956) or fluorometry when the amount of porphyrin present was too small for spectrophotometric determination. For iron determination, samples of liver homogenate were digested with 6 ml of HNO_3 and 1 ml of $HClO_4$ at 200° until clear (approximately 10 minutes), and iron was determined using a Perkin-Elmer Model 403 atomic absorption spectrophotometer with $FeCl_3$ as a standard. A portion of each liver was fixed in buffered neutral formalin, paraffin embedded, and stained with hematoxylin and eosin and with Gomori's method for iron. Data were analyzed by Duncan's multiple range test.

RESULTS

Table 1 shows the porphyrin content of the livers of mice given weekly

oral doses of TCDD. There was no significant increase in porphyrin accumulation in mice given weekly oral doses of 1 or 5 µg/kg TCDD. However, all mice given 25 µg/kg x 4 TCDD showed a large accumulation of uroporphyrins in the liver (2,000-fold increase). The porphyrin in the livers of TCDD treated mice was 8- and 7-carboxyporphyrins (90% and 10% respectively). In contrast, normal livers contained primarily protoporphyrin with lesser amounts of 8-carboxyporphyrin and a trace of coproporphyrin. Nine of the eighteen mice treated with 25 µg/kg x 4 TCDD died 2-3 days before termination of the experiment. There was no significant difference in the porphyrin content of livers from mice which died before termination of the experiment (204 ± 44 µg/g) and those sacrificed later (317 ± 115 µg/g).

TABLE 1

EFFECT OF TCDD ON PORPHYRIN ACCUMULATION IN LIVERS OF C56BL/6 MICE

Treatment	Porphyrins			
	8-COOH	7-COOH	4-COOH	2-COOH
	µg/g			
Control	0.14 ± 0.04	T	T	0.24 ± 0.15
1 µg/kg x 4	0.48 ± 0.15	T	T	0.29 ± 0.25
5 µg/kg x 4	0.61 ± 0.22	T	T	0.24 ± 0.16
25 µg/kg x 4	264 ±64*	22 ± 5*	T	(N.D.-1.9,range)
150 µg/kg x 1	598 ±82*	41 ± 6*	N.D.	N.D.

C57BL/6 mice were dosed orally weekly for 4 weeks with 0, 1, 5 or 25 µg/kg TCDD and killed 1 week after the last dose. One group was dosed orally with 1 dose of 150 µg/kg TCDD and livers were obtained after death or sacrifice of moribund animals. T = trace, N.D.= not detected. A trace of 5- and 6-carboxyporphyrins were detected in livers of mice given 4 doses of 5 or 25 µg/kg TCDD; they were not detected in livers from control or other treated groups. Each value represents the mean ± S.E.
*Significantly different from controls, $P<0.05$.

A second group of mice was treated with a single oral dose of 150 µg/kg TCDD, and livers were obtained after death or sacrifice of moribund animals. The time of death ranged from 17-30 days, but our samples included only those mice which died or were sacrificed 21-25 days after treatment (12 animals). Uroporphyrin content of the liver was increased an average of 4,000-fold in these mice (246-1197 g/g liver, Table 1). Only 8- and 7-carboxyporphyrins could be detected. Macroscopically, the livers showed accentuation of lobuli.

TABLE 2

EFFECT OF TCDD ON HEPATIC ALA SYNTHETASE ACTIVITY

Treatment	ALA Synthetase nM/g/hr	Liver Weight g
Control	50 ± 4	1.52 ± 0.03
1 µg/kg x 4 TCDD	60 ±11	1.80 ± 0.05*
5 µg/kg x 4 TCDD	43 ± 5	1.93 ± 0.04*
25 µg/kg x 4 TCDD	112 ±26*	1.62 ± 0.06

Animals were treated as described in Table 1. Each value represents the mean ±S.E.
*Significantly different from controls, $P<0.05$.

The activity of ALA synthetase was increased 2-fold by 4 weekly doses of 25 µg/kg TCDD (Table 2). No significant effect was seen at lower doses. These results agree with the effects of TCDD on porphyrin accumulation, i.e., the highest dose increased both porphyrin accumulation and ALA synthetase activity, but no effects were seen at lower doses. In contrast, liver weight was significantly increased by 4 weekly doses of 1 or 5 µg/kg but not by 25 µg/kg.

TABLE 3

EFFECT OF TCDD ON LIVER IRON CONTENT

Treatment	Liver Iron Content µg/g
Control	71 ± 5
25 µg/kg x 4 TCDD	114 ± 7*
150 µg/kg x 1 TCDD	187 ± 6*

Mice were treated as described in Table 1. Values are means ± S.E.
*P<0.05.

Total iron content of the liver was determined in mice treated with 25 µg/kg x 4 TCDD and 150 µg/kg x 1 TCDD. Total liver iron was increased in both groups of TCDD treated mice (Table 3).

DISCUSSION

Our results with C56BL/6 mice show that TCDD is porphyrogenic. A 4,000-fold increase in uroporphyrin content of the liver was seen as early as 21 days after a single dose of 150 µg/kg TCDD or after weekly administration of 25 µg/kg TCDD for 4 weeks. The porphyrin which accumulated in the livers of TCDD treated mice was identified as uroporphyrins (8- and 7-carboxyporphyrins). ALA synthetase was increased 2-fold by 25 µg/kg TCDD for 4 weeks but not by lower doses.

Light microscopic study of livers of mice treated with a single dose of 150 µg/kg TCDD revealed severe liver damage, particularly in the centrolobular areas where degenerated and necrotic hepatocytes were frequently observed. Cellular infiltrations (predominantly lymphocytes and polymorphonuclear leucocytes) were often observed in the damaged areas, together with ceroid pigment deposits. There was no evidence of hemosiderosis. There

was severe diffuse lipid accumulation (Oil red O stain) and enlargement of liver cell nuclei. Proliferation of bile ducts and bile duct epithelial cells was observed in the periportal areas. Marked liver damage was observed in mice treated with 25 µg/kg x 4 TCDD, and inflammatory cell infiltrates were seen quite often. Slight centrolobular degeneration and cellular infiltrations also occurred in mice treated with 5 µg/kg x 4 TCDD.

Since severe (150 µg/kg x 1 TCDD) or marked (25 µg/kg x 4 TCDD) liver damage was present at doses which resulted in porphyria, it is uncertain whether the porphyria and liver damage can be disassociated. In addition to liver damage, there was severe atrophy of the thymus in TCDD-treated mice, and edema and terminal hemorrhages were frequently observed (Vos et al., 1973).

Hemosiderosis has long been noted in the livers of patients with porphyria cutanea tarda (Uys and Eales, 1963). Hemosiderin and porphyrins have also been noted in livers of rats fed PCB's (Kimbrough et al., 1972). Kushner (1972) reported effects of iron on uroporphyrin III cosynthetase and suggested that accumulation of iron could be causally related to some porphyrias. We found only a moderate increase in the total iron in the livers of mice treated with high doses of TCDD (60% at 25 µg/kg x 4 and 160% at 150 µg/kg x 1) and no evidence of hemosiderosis histologically. In contrast, liver iron is increased 100-fold in Africans with advanced hemochromatosis (Gilman et al., 1945). Liver weights of mice given 4 doses of 25 µg/kg TCDD were normal, despite the fact that liver weight was increased 27% by a lower dose of TCDD (5 µg/kg x 4). The moderate increase in total liver iron may have been related to the severe liver damage rather than the porphyria and should be investigated further.

A preliminary report by Woods (1973) suggested that single doses of TCDD had no effect on hepatic ALA synthetase or liver fluorescence in the male rat,

mouse or guinea pig. Negative results with the male rat should not be unexpected, as the male rat is more resistant to the porphyrogenic action of many chlorinated compounds than the female rat (San Martin de Viale, 1970). Our results show that in the C56BL/6 mouse both induction of ALA synthetase and porphyria occur after 4 weekly doses of 25 µg/kg TCDD or a single lethal dose of 150 µg/kg. Species, sex and strain differences may play an important role in susceptibility to porphyrogenic effects of TCDD and should be investigated. Secondly, in contrast to allylisopropylacetamice, which produces porphyrinuria and induction of ALA synthetase a few hours after administration(De Matteis and Prior, 1962, Marver et al., 1966a), many chlorinated compounds such as hexachlorobenzene and polychlorinated biphenyls produce porphyria and increase ALA synthetase only after several weeks exposure (Ockner and Schmid, 1961; Rajamanickam et al., 1972; Goldstein et al., 1973). A dose response for the porphyrogenic action of TCDD should be determined after longer exposure, since effects may be seen at lower doses after longer periods of exposure.

Our results show TCDD to be the most potent compound known to produce porphyria in mammals. As reported for the chick embryo (Poland and Glover, 1973), TCDD is approximately 3 orders of magnitude more potent than other known porphyrogenic chemicals. Aroclor 1254, a mixture of PCB's, is porphyrogenic when fed to rats at levels of 100 ppm (approximately 10 mg/kg/day) for 4 months (Goldstein et al., 1973). Two European PCB samples have been reported to be contaminated with small amounts of tetrachlorodibenzofuran (Vos et al., 1970). The potency of TCDD in producing hepatic porphyria suggests that even slight contamination of environmental chemicals with TCDD or chlorinated dibenzofurans may contribute substantially to the porphyrogenic effects of these chemicals. Results from feeding studies on

chemicals which may be contaminated with chlorinated dibenzodioxins or dibenzofurans should be interpreted with caution.

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